Studies on *"encephale isolé"* and *"cerveau isolé"* give reason to infer that during the process of Nivalin's exciting effect on the cerebrum, its influencing of the cholinergic neurons of the midbrain's reticular formation feasibly plays a large role.

Past experimental observations as well as [illegible] experiences are to be construed as Nivalin acting both on all links of the reflex arc as well as the neuromuscular synapses.

II. Clinical Observations

Nivalin's therapeutic range of application above all encompasses diseases of the neuromuscular apparatus – myasthenia gravis pseudoparalytica, dystrophia musculorum progressiva, etc. – and diseases of the peripheral motoric neuron – pareses and paralyses following poliomyelitis, neuritis and polyneuritis; Nivalin is also used at times to treat some diseases which affect the central motor neuron – infantile cerebral palsy, multiple sclerosis, hemiplegias subsequent insults to the brain, traumatic damage to the central nervous system.

We treated 108 patients (together with I. Georgiev, W. Daschin, B. Jordanov, A. Samardjiev and W. Nikolkov) of the myopathy group. Among them were 19 patients with myasthenia gravis pseudoparalytica who exhibited good success; all patients were more or less positively influenced. In 9 cases, the effect was noted following the first injection of 2.5 mg. An even clearer and more consistent effect (up to 3 hours) could be noted after increasing the doses. At the same time, an obvious improvement in motor functioning subsequent Nivalin's direct period of action was also observed. In the case of 8 patients, no direct effect was determined following an initial dose of 2.5 mg; an effect only occurred after 4 to 10 days and it was of similar duration to that of the cited 9 cases. Only in 2 cases was just one direct effect observed, persisting in the case of one of the patients for 2 hours, for the other 30 minutes. Four of the total 19 patients were strongly influenced – up to the complete restoration of motoricity, another 10 were substantially and five slightly influenced. A fact to be particularly emphasized is that the effect of the Nivalin treatment also persisted after cessation of the two-month regimen. This relates to two cases in which an improvement in motor functioning was also observed after the period of direct action; in the first case, the Nivalin effect was sustained for 4 months, in the second for 10 months following treatment. In five cases, the Nivalin effect was initially estimated, a combined treatment of Nivalin and prostigmin was thereafter administered, whereby the preparations were either given at the same time or successively. Administering the two medicines resulted in a potentiation of action, whereby a better effect ensued at lower doses and no adverse side effects occurred.

The comparative assessment of Nivalin and prostigmin action in cases of myasthenia gravis pseudoparalytica shows that the Nivalin effect had slower onset – up to 30 minutes post injection – yet was sustained longer – up to 3-4 hours. In contrast to the prostigmin effect, which is known to only be apparent when present in the organism, Nivalin also exerts an effect thereafter, one which is not so pronounced, but which in some cases can be sustained for months following treatment.

Our observations with regard to progressive muscular dystrophy are extremely interesting and promising. Of 64 cases treated, 19 patients experienced great improvement. This was expressed in new motor activities which had been impossible prior to beginning the treatment, in some cases walking as well as sitting and standing stamina was restored, and a considerable increase in the volume, speed and strength of the motorics was observed. We observed improvement in another 19 patients – considerable increase in motoricity without incident of any new motorics. Slight improve-

ment – small increase in volume of movement – occurred in 15 cases. In the case of the remaining 11 patients, no visible change could be noted. We conducted a comparative observation of the curative effect at relatively low doses as proposed by M. D. Maschkowski and at 2-3 times higher doses based on our own proposed regimen on a group of 24 children (together with A. Samardjiev and W. Nikolkov). The results were as follows: of a total 18 children treated with low doses, 5 exhibited an unchanged condition, slight improvements were observed in 7, one improved, and there were no cases of strong improvement. The group of children treated at higher doses exhibited the reverse; i.e., of a total of 11 cases in which no toxic symptoms were exhibited, all were influenced, whereby 3 were slightly improved, 4 generally improved and another 4 improved considerably. These children still remain under observation today and are given a two-month Nivalin treatment at intervals of 2-6 months. We are of the impression that the results achieved are sustained for 3-6 months on average and each new treatment period continues to further improve the condition of the patients. In this regard, the ascending and the ascending-pseudohypertrophic form shows better influencing than the descending form.

As far as the other diseases of the myopathia group, Nivalin brought on the following results: of a total of 18 patients with Amyotrophia spinalis and Amyotrophia neuralis, 6 improved and 10 were uninfluenced; of a total of 5 patients with myotonic dystrophy, one case showed marked improvement and two others showed general improvement, whereby the myotonic component was scarcely affected; the remaining two were unchanged; of a total of 4 patients with congenital myotonia, marked improvement was observed in one, slight improvement in another, the other two remained uninfluenced.

Nivalin has the most favorable effect on diseases involving the peripheral motor neuron. First to mention here is poliomyelitis, both its recuperative stage as well as its late residual stage. D. Paskov emphasized the effectiveness of immediate treatment. He gained his experience in the I. Infectious Hospital in Sofia where a group of 21 children with severe paralytic forms was treated. The cases were grouped pursuant to the Kendall classification as follows: 12 in Group "O" – complete absence of active movement of the muscles concerned; 7 in Group I – insignificant excitation of the tendons of the relevant muscles when attempting to perform an active movement; 3 in Group II – possible active movements of the relevant muscles without exertion. Treatment commenced as of the 13th up to the 69th day subsequent the onset of the disease. 11 cases were the spinal form, 8 were bulbar, and 2 the bulbospinal form. Improvement in motor functioning occurred very rapidly – from the 5th to the 20th day after beginning treatment with Nivalin. Eighteen children exhibited some varying degree of improvement or a complete recovery and only 3 cases remained uninfluenced. Alexander Levy (Heliopolis) reported on 16 children aged 9 months to 9 years old whom he treated with Nivalin for at least 1.5 years subsequent the onset of the disease. He was able to completely cure one case from Group I of the Kendall classification while remarkable results were recorded for 15 cases from Group II and III of the Kendall classification. He stresses the rise in local temperature in the extremities concerned and the increase in muscle tone as well as the volume, speed and strength of the movements. In his opinion, the increased local temperature attests to Nivalin's positive effect on the circulation.

K. Descovich (Bologna) treated 5 children aged 3-6 years old with severe paralyses and tropic disorders in accordance with the acute stage of poliomyelitis with Nivalin. All cases were positively influenced and new movements occurred to such an extent that the disabled children were able to stand up and to walk. The tropic ulcers were improved. N. Syazeh (Buenos Aires) observed 3 cases in the residual stage of poliomyelitis in which movements occurred in paralyzed muscles and the trophism was positively affected under the influence of Nivalin. N. Bilimov likewise reported on a case in the residual stage of poliomyelitis in which he observed a 50-day treatment with Nivalin healing tropic ulcers which had been resistant to all other therapeutic measures.

Even more positive are the findings from the treatment of neuritises; 100 cases in all (together with L. Georgiev, W. Daschin, B. Jordanov), predominantly neuritis facialis. We observed 44 cases, the majority of which were deemed *"a frigore"* from the etiological standpoint. In half the cases, treatment began during the first month. At first, we waited for the inflammatory period to subside, later Nivalin was also given during this period. Nine cases recovered fully, 27 improved considerably, 6 were slightly influenced and 2 remained unchanged. The best results were to be noted in fresh cases, but Nivalin is also indicated in cases dating back even several years. Incidents of contractures and pathological synkinesia were not observed during the Nivalin treatment; in 2 cases, previously long-standing contractures diminished.

Of 14 cases of neuritis n. oculomotorii, 6 recovered fully, 4 improved substantially, another 4 remained uninfluenced. Of 6 cases of various mononeuritis (n. n. peroneus, radialis, recurens), 3 were cured, 2 showed good improvement and 1 case remained unchanged.

The effect of Nivalin on neuritises can thus be summarized as follows: 80% of all neuritises were either completely cured or vastly improved, 9% improved slightly, 11% remained unchanged.

In 20 polyneuritis cases in which an inflammatory or toxic etiology was determined, the following results were obtained: 6 recovered fully, 5 were improved, another 5 improved slightly and 4 remained uninfluenced. We made an interesting observation in a case of a polyneuritis as a result of [illegible] toxication: the patient recovered fully following a 50-day Nivalin treatment.

The findings from treating diseases involving the central motor neuron, a total of 64 cases in all (together with I. Georgiev, W. Daschin and B. Jordanov), are less straightforward. We treated 22 children aged 4-14 years with Nivalin (together with A. Samardjiev and W. Nikolkov). These were cases of infantile cerebral palsy (Morbus Little) of prenatal, natal and postnatal etiology. Improvement manifested in diminished spasticity, intensification of existing active movements and occurrence of new ones, suppression of hyperkinesia, and stimulation of cognitive mnestic activity. Strong improvement was observed in 4 cases, 9 cases showed general improvement, 6 cases experienced slight improvement, 3 children remained uninfluenced. In terms of the clinical forms, the best results were noted for the diplegic form (11 children improving to varying degrees and 1 case remaining unchanged). In the hemiplegic, hypotonic and extrapyramidal forms, improvements occurred in approximately half the cases. Of the individual symptoms, spastic paresis and cognitive mnestic function showed the best response.

Of 26 patients with multiple sclerosis, 20 of which were chronic severe forms, 4 cases showed improvement in the spastic paralysis while coordination impairments were less affected. These findings do not concur with the findings made by G. Nastev and peers; they observed improvement in 20 out of 25 predominantly fresh cases.

Out of 11 patients with amyotrophic lateral sclerosis, a certain positive influence could only be determined in one case. The cases presenting [illegible] did not tolerate [illegible] high doses of Nivalin. G. Nastev and peers reported a favorable influence in 7 mild cases in which the effect occurred after a longer application of higher doses.

Of 11 patients suffering impairments following insult to the brain, in which treatment began 9 months following onset of the disease, 5 cases exhibited substantial improvement with respect to spastic hemiparesis, general improvement was observed in 2 cases, another 2 showed slight improvement and 1 case was uninfluenced. N. Kilimov published similar results. In 10 transverse myelitis cases and in some other cases of damages to the central motor neuron, results were less satisfying.

III. Indications and Contraindications Side Effects

As can be seen from the clinical material presented, indication for Nivalin treatment is primarily given in cases of damage to the peripheral motoric neuron due to poliomyelitis, neuritises and polyneuritises as well as in cases of myopathia – predominantly myasthenia gravis pseudo-paralytica. Results are particularly promising in cases of progressive muscular dystrophies. Nivalin exhibits a good effect against some diseases involving the central motor neuron, e.g. infantile cerebral palsy; in the case of multiple sclerosis, the postapoplectic paralysis Nivalin must be given under stricter attention to the course of treatment since a number of cases did not exhibit a positive effect until after several weeks. There are certain differences of opinion – particularly in older publications – as to the effect of Nivalin treatment, mainly attributed to the use of the relatively lower doses as proposed by M. D. Maschkowski. More recent observations with the 2-4 times higher doses we propose (V. Bergamini, P. Baggiore, N. Kilimov) returned results which [illegible].

Contraindications for Nivalin treatment are: cardiac decompensation, bradycardia, bronchial asthma and epilepsy. It was initially assumed that extrapyramidal hyperkineses belonged among the contraindications against Nivalin treatment, yet our later experience indicated that these disorders were positively influenced in a number of cases, whereby no exacerbation of the condition was ever observed. E. F. Kulkowa-Dawidenkowa (Moscow) is of the opinion that Nivalin can also be used in cases of infantile cerebral palsy with epileptic seizures, whereby this treatment should be combined with anticonvulsant medication.

Side effects occur relatively rarely. We never observed any adverse symptom which would have necessitated the administration of atropine, the Nivalin antagonist. At times, headache, dizziness, cardiac oppression, hypersalivation, nausea, vomiting, abdominal pain, diarrhea or arterial hypotension were observed 30 to 60 minutes post injection. All these incidents were short-lived and subsided after the patient lay down in bed. Should they be more strongly pronounced, the dosage must be reduced by 2.5 to 5 mg without interrupting the treatment. It will generally be possible to again increase the dosage within a few days without experiencing any side effects. Cases of Nivalin intolerance by individuals are extremely rare.

IV. Application and Dosage

Nivalin is given subcutaneously. We have recently demonstrated (together with Kr. Athnassov, St. Stanev and L. Neprienkowa) that it can also be introduced into the organism by ionophoresis.

The subcutaneous administration begins at low initial doses of 1.25-2.5-5 mg with the dose being gradually increased by the same amount – taking individual reactivity, which differs greatly, into consideration – every third to fifth day until a dose of 15-30 per day is reached. The daily dose is given 2-3 times at intervals of 12 or 6 hours with 5-10 mg per dose being injected. Children also tolerate higher doses well. They can even be given 20-30 mg per day, naturally under the strict evaluation of individual tolerance, which necessitates great vigilance when increasing the doses.

Upon evaluating the findings on Nivalin's curative effect, we must reiterate that it does not act on the cause of an illness but rather changes its pathogenetic mechanism, whereby it facilitates both the conduction of the impulses in all links of the nervous system as well as their transmission to effector organs. Nivalin thereby lays claim to only completely healing a relatively large share of neuritises and the motor disturbances of poliomyelitis. In all other cases involving myopathies, infantile cerebral palsies, hemiplegias subsequent insults to the brain (i.e., patients usually constituting invalids to some degree or another), Nivalin only effected a more or less pronounced improvement during its present study period.

Every clinician is aware of the thankless task this patient therapy represents, the recognition received for each – even the slightest – improvement, and the hope this improvement instills.

A further task associated with rehabilitation – once the Nivalin has brought about an improvement – is to help a great many of these patients assume socially useful activities.

The boundaries of Nivalin's therapeutic possibilities have not yet been drawn. The problems associated with its new areas of application cannot be resolved until more clinical material has been amassed.

[Abstract printed in German, Greek and English]

Nivalin (Galanthamin) is a new drug, inhibiting cholinesterase with a wide field of action. It is a tertiary amine, resembling eserine, but is much less toxic. It is distinguished from prostigmin, which is a quaternary amine, chiefly by the duration of its action.

Nivalin has a very good curative effect upon diseases of the neuromuscular apparatus – myasthenia gravis pseudoparalytica, dystrophia musculorum progressiva, etc. – and upon lesions of the peripheral motoric neuron, the residual stage of poliomyelitis, neuritis (above all neuritis n. facialis), polyneuritis, etc. In many cases also a good influence upon lesions of the central motoric neuron was observed, such as cerebral poliomyelitis, multiple sclerosis, hemiplegias after insults, myelitis, etc.

Nivalin is applied subcutaneously in doses increasing up to 10-25 mg per day. The drug is very well tolerated, side effects are rare.

Literature

[see original for names and citations]

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DIFFERENTIAL ALTERATIONS IN MUSCARINIC RECEPTOR SUBTYPES IN ALZHEIMER'S DISEASE: IMPLICATIONS FOR CHOLINERGIC-BASED THERAPIES

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Summary

Molecular subtypes of muscarinic receptors (m1-m5) are novel targets for cholinergic replacement therapies in Alzheimer's disease (AD). However, knowledge concerning the relative distribution, abundance and functional status of these receptors in human brain and AD is incomplete. Recent data from our laboratory have demonstrated a defect in the ability of the M1 receptor subtype to form a high affinity agonist-receptor-G protein complex in AD frontal cortex. This defect is manifested by decreased M1 receptor-stimulated GTP-γS binding and GTPase activity and by a loss in receptor-stimulated phospholipase C activity. Normal levels of G proteins suggest that the aberrant receptor-G protein interaction may result from an altered form of the m1 receptor in AD. The combined use of radioligand binding and receptor-domain specific antibodies has permitted the re-examination of the status of muscarinic receptor subtypes in the human brain. In AD, normal levels of m1 receptor [^{3}H]-pirenzepine binding contrasted with diminished m1 immunoreactivity, further suggesting that there is an altered form of the m1 receptor in the disease. Reduced m2 immunoreactivity was consistent with decreased numbers of m2 binding sites. Increased levels of m4 receptors were observed in both binding and immunoreactivity measurements. These findings suggest one possible explanation for the relative ineffectiveness of cholinergic replacement therapies used to date and suggest potential new directions for development of effective therapeutic strategies for AD.

Key Words: muscarinic receptor subtypes, Alzheimer's disease, cholinergic replacement therapies

Application of biochemical and molecular approaches to the study of Alzheimer's disease has provided some clues to its possible progression and pathogenesis. However, one of the greatest challenges to as well as on-going controversies among investigators in the field, results from attempts to set the morphological and biochemical changes into a temporal sequence of pathogenesis. Knowledge of the pathogenic cascade is necessary for the rational design of therapeutic interventions.

Cholinergic Neurotransmission: Focus of Therapeutic Interventions

Alzheimer's disease is characterized by cerebral cortical atrophy, neuronal loss, intraneuronal neurofibrillary tangle formation and the widespread deposition of extracellular parenchymal and cerebrovascular β-amyloid (βA; 1). Neurochemical deficits in presynaptic cholinergic markers contribute significantly to the development of Alzheimer's disease symptomatology (2, for review). The precise relationship between the hallmark neuropathologic and neurochemical features of Alzheimer's disease is not clear, at present. Cholinergic neurochemical deficits suggested a relatively selective vulnerability of the cholinergic system, and served, along with the demonstrated involvement of the cholinergic system in learning and memory processes, as the basis for the cholinergic hypothesis of Alzheimer's disease. The cholinergic hypothesis provided the impetus to the pharmaceutical industry for the design of cholinergic replacement therapies akin to the use of L-DOPA in the symptomatic treatment of Parkinson's disease.

Over the past ten years our laboratory has studied the role of the cholinergic system in the pathogenesis and progression of Alzheimer's disease. We have specifically focused on the muscarinic acetylcholine receptors since they are the primary targets of any cholinergic replacement therapies. Treatment strategies to date have included acetylcholine precursors and release enhancers, acetylcholinesterase inhibitors and direct acting cholinergic agonists. Tetrahydroaminoacridine (THA), a cholinesterase inhibitor, has recently been approved for clinical treatment of mild to moderate Alzheimer's disease (3). However, the narrow benefit-to-risk profile of THA, has limited its usefulness (4). Data from our laboratory demonstrating a complex pharmacological profile for THA (5), including actions as both a competitive and allosteric antagonist at the muscarinic receptor, ion-channel-directed effects, and its ability, like other cholinesterase inhibitors, to down-regulate muscarinic receptors, suggest that THA's multiple actions may also contribute to its limited efficacy.

Muscarinic Receptors in Alzheimer's Disease: Early Studies

Cholinergic replacement strategies are based on the assumption that the number and functional integrity of postsynaptic receptors are relatively unchanged in Alzheimer's disease. Early studies concerning the number of muscarinic receptors in the Alzheimer's disease brain provided equivocal results (6). However, in 1985 we re-examined the status of muscarinic receptors in Alzheimer's disease in light of emerging evidence for at least two distinct populations of sites (M1 and M2) in the brain with different affinities for agonists (7). Our studies demonstrated a selective loss of pre-synaptic M2 sites and a relative sparing of the post-synaptic M1 sites in Alzheimer's disease (8). These findings suggested that non-receptor subtype selective therapies may not be beneficial since their action at pre-synaptic autoreceptors may inhibit further acetylcholine release. Thus, the pharmaceutical industry began to focus on the development of M1-selective agonists and M2 receptor antagonists.

Muscarinic Receptors: Viable Therapeutic Targets in Alzheimer's Disease?

Emphasis began to shift away from single cholinergic neurotransmitter replacement for the treatment of Alzheimer's disease for several reasons: first, the overall lack of efficacy of most cholinergic treatment strategies; second, the demonstrated involvement of a number of other cortical and subcortical neurotransmitter systems; and third, the concept that the overproduction and accumulation of βA may play a pathogenic role in the disease. Our laboratory investigated an alternative explanation: that the limited efficacy of cholinergic therapies may be due to dysfunctional receptor response mechanisms in the disease.

Muscarinic receptors are members of the large family of structurally related membrane-

spanning proteins and serve to translate the extracellular binding of acetylcholine into an intracellular response by interacting with guanine nucleotide binding or G proteins. Consequently, disruption of receptor-G protein interactions prevent cellular activation. Receptors coupled to or uncoupled from their G proteins can be differentiated by the binding of agonists with high and low affinities, respectively. Under conditions that permitted the assay of agonist affinity states of the M1 receptor (labeled with the M1-selective muscarinic antagonist [^{3}H]-pirenzepine), we demonstrated that M1 receptors in Alzheimer's disease frontal cortical samples lost the ability to form high affinity, guanine nucleotide sensitive agonist-receptor-G protein complexes (9).

Autoradiographic mapping of high and low affinity carbachol binding to [^{3}H]-pirenzepine-labeled M1 receptors was performed in slide-mounted coronal sections of human brain to determine the regional specificity of the defect in M1 receptor agonist binding. In age-matched control subjects, carbachol displaced [^{3}H]-pirenzepine binding in the frontal cortex and hippocampus (Fig. 1, left-hand panels), and also in entorhinal cortex (area 28; data not shown). The binding of [^{3}H]-pirenzepine was recovered when high affinity agonist sites were uncoupled with guanine nucleotide. In corresponding Alzheimer's disease brain regions, carbachol was unable to displace [^{3}H]-pirenzepine binding and there was no effect of guanine nucleotide (Fig.1, right-hand panels). However, in the visual cortex (Fig. 1), and also in the primary motor cortex (area 4) and the putamen (data not shown), guanine nucleotide-sensitive, high affinity agonist binding was spared in Alzheimer's disease, consistent with regions known to be relatively unaffected in the disease. Thus, the loss in high affinity agonist binding to M1 receptors is regionally specific.

Deficits in Muscarinic Receptor-mediated Functional Responses

The apparent loss of cortical muscarinic receptor functional integrity was further investigated by examining the ability of muscarinic agonists to stimulate G protein and effector function. Receptor (agonist) stimulated [^{35}S]-GTPγS binding, GTPase activity and phospholipase C activity in Alzheimer's disease frontal cortical samples were significantly diminished compared to control samples (Fig. 2). Losses in receptor-stimulated activities in Alzheimer's disease did not result from losses in the number of G proteins (10,11) or in basal G protein (11) or phospholipase C (12) functions. The loss in receptor-mediated responses in Alzheimer's disease demonstrated regional-specificity, whereby reductions were observed in the frontal cortex but not in occipital cortex or putamen (11). The degree of receptor-stimulated increases in GTPγS binding and GTPase and PLC activities were positively correlated with M1 receptor high affinity agonist binding (K_L/K_H ratio) in control samples. However, a similar correlation was not observed for Alzheimer's disease samples, presumably resulting from the greatly diminished or complete loss of receptor-stimulated activities. This correlation provided additional evidence that the guanine nucleotide sensitive, high affinity agonist binding is a good indicator of the functional integrity of the receptor.

Receptor Subtype-specific Alterations in Alzheimer's Disease

Recent molecular cloning of the genes encoding muscarinic receptors has demonstrated that the originally postulated pharmacologically-defined subtypes (M1-M3) consist of at least five (m1-m5) distinct primary sequences (13). Characterization of the molecular subtypes has been hampered by the lack of sufficiently selective ligands to distinctly label each of the receptor proteins. Therefore, while the differential involvement of subclasses of muscarinic receptors in Alzheimer's disease was apparent, it was not known how the underlying molecular subtypes were altered in the disease or which subtypes remained as viable targets for cholinergic drugs. This fact has limited drug development for the symptomatic treatment of Alzheimer's disease.

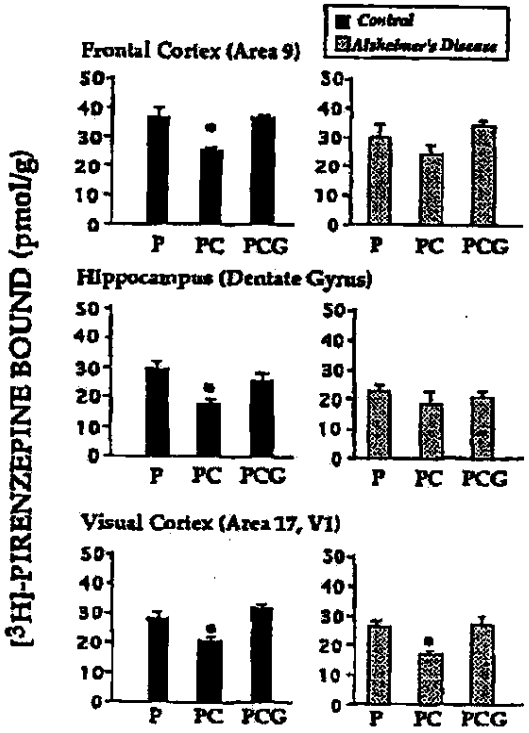

Figure 1. Quantitative autoradiographic mapping of the regional effects of guanine nucleotides on agonist binding to the M1 muscarinic receptor. Slide-mounted adjacent sections of the human brain were labeled with 3 nM [^{3}H]-pirenzepine alone (P), or in the presence of 10 μM carbachol to occlude high-affinity agonist sites (PC), or in the presence of carbachol plus 0.2 mM GppNHp to "uncouple" high affinity agonist binding (PCG). Region-of-interest densitometric measurements were made within representative areas. A significant decrease in [^{3}H]-pirenzepine binding ($p < 0.001$) in the presence of carbachol (PC) demonstrates the presence of high affinity agonist sites. The failure of carbachol to decrease [^{3}H]-pirenzepine binding in some AD brain regions demonstrates the loss of high affinity agonist binding to the M1 receptor.

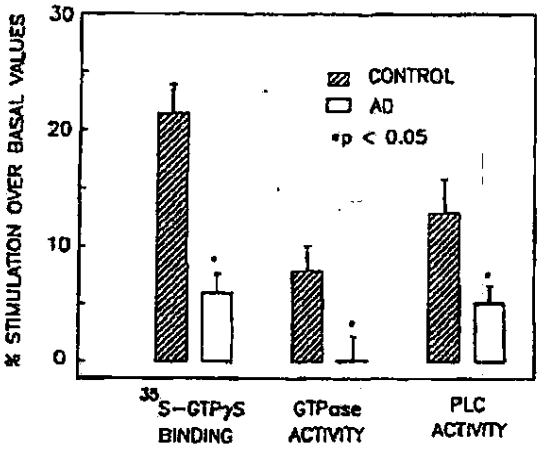

Figure 2. Loss in muscarinic receptor - mediated functional responses in Alzheimer's disease frontal cortex. Data are expressed as percentage increase over basal (absence of agonist) values in the presence of 1 mM carbachol. [^{35}S]-GTPγS binding, [γ^{32}P]-GTP hydrolysis and PLC activity were measured as described elsewhere (11,12).

Therefore, we devised a novel, combined equilibrium/kinetic binding approach to selectively label the m1-m4 receptor subtypes (14,15). The method takes advantage of the partial receptor subtype selectivity of the muscarinic antagonists pirenzepine, guanylpirenzepine and AF DX-116 by equilibrium binding and the distinct kinetic binding properties of the individual receptors for the muscarinic antagonists N-methylscopolamine (NMS) and dexetimide, muscarinic antagonists

that demonstrate little or no receptor subtype selectivity in equilibrium binding assays. The subtype-selective labeling conditions were validated in CHO cells expressing the m1-m5 receptor proteins (Fig. 3).

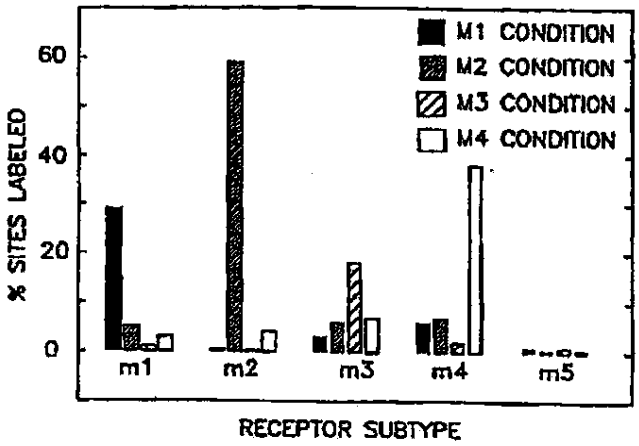

Figure 3. Muscarinic receptor-subtype selective labeling conditions in m1-m5 transfected CHO cells. Data are expressed as the percentage of sites labeled by each receptor labeling condition: M1: 3 nM [^{3}H]-pirenzepine for 60 min; M2: 0.3 μM pirenzepine for 60 min followed by 2 min incubation with 0.5 nM [^{3}H]-NMS; M3: 0.5 nM NMS for 5 min, then 60 min incubation with 0.5 nM [^{3}H]-NMS followed by 60 min dissociation in presence of 1 μM atropine; M4: 10 nM dexetimide for 30 min, then 15 min incubation with 0.5 nM [^{3}H]-NMS plus 200 nM guanylpirenzepine and 250 nM AF DX-116 followed by 5 min dissociation with 1 μM atropine (14,15).

These labeling conditions were applied to frontal cortical samples from control and Alzheimer's disease brains to determine the levels of the m1-m4 receptors (Table I). The unchanged level of m1 receptors and diminished levels of m2 receptors were consistent with our earlier findings. However, while the level of the m3 receptor was apparently unchanged, there was a significant increase in the level of m4 receptors. The level of m4 receptors has not been previously addressed in Alzheimer's disease. Their upregulation provided a possible explanation for the conflicting results in earlier studies regarding the levels of total and M1 receptors measured using non-receptor subtype-selective radioligands.

TABLE I

MOLECULAR MUSCARINIC RECEPTOR SUBTYPES IN ALZHEIMER'S DISEASE FRONTAL CORTEX: COMPARISON OF LIGAND BINDING AND i3-LOOP-SPECIFIC ANTIBODY REACTIVITIY

SUBTYPE	BINDING	IMMUNOREACTIVITY
m1	no change	35-50% ↓
m2	50-90% ↓	30-50% ↓
m3	no change	no change
m4	30-50% ↑	35-40% ↑
m5	n.d.	no change

In parallel to the binding studies, we have also used a panel of receptor subtype specific antibodies (16) to determine the level and expression of m1-m5 receptors in Alzheimer's disease (see Table I). Polyclonal antibodies were raised against fusion proteins corresponding to the putative third cytoplasmic (i3) loop of the m1-m5 receptors and the receptor-specific antisera were used in quantitative immunoprecipitation assays (17). In Alzheimer's disease brains, decreased levels (35-50%) of m1 receptor immunoreactivity were observed in frontal, parietal and temporal cortical regions and hippocampus. This finding contrasted with the previously measured unchanged levels of m1 receptor binding and suggested that while the number of m1 receptor binding sites or proteins is unchanged, there is an altered form of the receptor protein resulting in reduced antibody recognition. The antibodies recognize an epitope in the i3 loop of the receptor which is critical for receptor-G protein interaction. The receptor domains involved in antagonist binding, namely the transmembrane regions, are distinct from the i3 loop and thus, antagonist binding and receptor-G protein interaction may be differentially modulated (18). It is not known at present how the m1 receptor i3 loop may be altered in Alzheimer's disease. Nonetheless, these findings suggest that the m1 receptor subtype may not be a suitable therapeutic target, particularly in later stages of the disease. The results also demonstrate that antagonist binding studies do not provide complete information concerning receptor function or structure (see Table II).

TABLE II

EVIDENCE FOR M1 MUSCARINIC RECEPTOR - G PROTEIN UNCOUPLING IN ALZHEIMER'S DISEASE
1. ↓ High affinity agonist binding[9,19,20]
2. ↓ Receptor-stimulated PLC activity[12,21]
3. ↓ Receptor-stimulated GTPγS binding[11,22]
4. ↓ Receptor-stimulated GTPase activity[11]
5. ↓ m1-receptor i3-loop immunoreactivity[17]

The reduction in immunoprecipitated m2 receptor protein was consistent with previous demonstrations of decreased M2 receptor binding sites and was in keeping with the degeneration of basal forebrain cholinergic neurons and the presynaptic location for at least a portion of the cortical and hippocampal M2 receptor on cholinergic nerve terminals. Consistent with the increased levels of M4 receptor binding sites, m4 receptor immunoreactivity was increased in regions parallel to those showing diminished m1 receptor immunoreactivity. The opposite modulation of the m1 and m4 receptor subtypes in Alzheimer's disease is a novel finding. This differential regulation of m1 and m4 receptors was not previously demonstrated because most earlier studies used radioligands with overlapping affinities for the m1 and m4 subtypes. At this time, it is uncertain whether the neuroadaptive increase in m4 receptors is related to the disease process. Reported evidence that the M4 receptor subtype may presynaptically modulate release of some neurotransmitters (23,24) suggests that this subtype may be a valuable, new therapeutic target. Also, since there are relatively low levels of m4 receptors in most peripheral tissues (25), drugs aimed at this subtype maybe free of many of the side-effects associated with other cholinomimetic drugs. There were no apparent alterations in the level of m3 or m5 receptor immunoreactivities in Alzheimer's disease

Implications for Receptor Subtype-specific Alterations in Alzheimer's Disease

Information about the functional status of muscarinic receptor subtypes may have important implications beyond cholinergic replacement therapies directed at receptor binding sites. Considerable evidence supports the hypothesis that βA plays an early and possibly causative role in the neurodegenerative process associated with Alzheimer's disease (26). The hypothesis is based on demonstrated alterations in APP proteolysis and expression resulting from mutations in the APP gene and from advanced age and head injury, two of the major risk factors for the disease. However, the precise molecular mechanisms leading to the deposition and accumulation of insoluble amyloid plaques still remain unclear. Recent studies have demonstrated that non-amyloidogenic processing of APP is enhanced, and βA generation diminished, by m1 specific muscarinic receptor-mediated activation of protein kinase C, suggesting a relationship between APP processing and muscarinic receptor activation (27,28). This suggestion is in keeping with the proposal put forward 13 years ago by Price and colleagues (29) that loss of neurons in the basal forebrain leading to reductions in cholinergic markers is associated with the development of senile plaques. A relationship between the primary neuropathological hallmark and one of the primary neurochemical deficit in Alzheimer's disease is provocative. Muscarinic receptor activation regulates neuronal PKC activity and intracellular Ca^{++} levels. Loss of cortical cholinergic activation, either via presynaptic cholinergic denervation or the loss of postsynaptic m1 receptor responsiveness, may lead to alterations in the usual receptor-regulated control of intracellular signalling. Disruption of Ca^{++} homeostasis and PKC aberrations are associated with increased vulnerability of neurons to excitotoxic insults and with alterations in the metabolic processing of APP (30). Since m1 receptors are localized, in part, to excitatory amino acid synapses (31), dysfunctional m1 receptors may result in compensatory increases in metabotropic responses to glutamate and lead to an increased accumulation of βA. Nevertheless, recent evidence for the role for apolipoprotein E gene dosage in determining the rate of Alzheimer's disease expression has dampened some of the enthusiasm for βA deposition as a primary event in the disease (32). Clearly, the precise steps in the pathogenic cascade are far from understood, but the data presented here suggest that differential regulation of muscarinic receptor subtypes may play a key role in the progression of the dementia associated with the disease. The possibility that disequilibrium in cholinergic neurotransmission, resulting from losses in cholinergic innervation and/or in cholinergic receptor responsiveness, may contribute significantly to progression of the disease should be considered. Greater understanding of the possible cause and effect relationships between the neurochemical and neuropathological hallmarks of the disease, as well as of the temporal sequence of neurodegenerative events, should aid in the search for more effective therapeutic strategies.

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EXHIBIT 44

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Colloquium Paper

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Muscarinic acetylcholine receptor expression in memory circuits: Implications for treatment of Alzheimer disease

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ABSTRACT Cholinergic transmission at muscarinic acetylcholine receptors (mAChR) has been implicated in higher brain functions such as learning and memory, and loss of synapses may contribute to the symptoms of Alzheimer disease. A heterogeneous family of five genetically distinct mAChR subtypes differentially modulate a variety of intracellular signaling systems as well as the processing of key molecules involved in the pathology of the disease. Although many muscarinic effects have been identified in memory circuits, including a diversity of pre- and post-synaptic actions in hippocampus, the identities of the molecular subtypes responsible for any given function remain elusive. All five mAChR genes are expressed in hippocampus, and subtype-specific antibodies have enabled identification, quantification, and localization of the encoded proteins. The m1, m2, and m4 mAChR proteins are most abundant in forebrain regions and they have distinct cellular and subcellular localizations suggestive of various pre- and postsynaptic functions in cholinergic circuits. The subtypes are also differentially altered in postmortem brain samples from Alzheimer disease cases. Further understanding of the molecular pharmacology of failing synapses in Alzheimer disease, together with the development of new subtype-selective drugs, may provide more specific and effective treatments for the disease.

Alzheimer disease (AD) is the most common cause of memory loss and dementia. Retarding or arresting disease progression is an important therapeutic goal, which at present is not possible because of limited understanding of the cause(s) of the disease. An alternative therapeutic goal of ameliorating some of the cognitive and behavioral problems may be closer to realization. Many recent advances in understanding the molecular pharmacology of the vulnerable transmitter systems in AD have provided the opportunity to develop improved pharmacological agents. Loss of synapses appears to be one of the most critical aspects of the final common pathway that leads to the dementia (1, 2), and neurochemical studies suggest that synapses containing acetylcholine (ACh), glutamate, and serotonin in neocortex and hippocampus are predominantly affected (3, 4). This brief review focuses on the cholinergic system and, in particular, the muscarinic ACh receptor (mAChR) molecules involved in modulation of memory-related synapses in the basal forebrain and hippocampus, and the therapeutic implications for AD.

Cholinergic Memory Circuits and AD

The basal forebrain contains a well characterized group of magnocellular cholinergic neurons extending from the medial septal region through the nucleus basalis of Meynert, Ch1–Ch4 (5), which provide the majority of cholinergic innervation to the hippocampus and neocortex. The septo-hippocampal component of this system is the best studied and derives from observations of the crucial role of the hippocampus in learning and memory, the early and pervasive loss of memory in AD, as well as the early and extensive hippocampal pathology in the disease (6). In fact, synapse loss in hippocampus and pathology of cholinergic basal forebrain neurons are the best predictors of memory impairment in AD (2, 7).

Several lines of evidence implicate impaired cholinergic neurotransmission at mAChR as contributing to the dementia symptoms in AD, including: (*i*) consistent depletion of choline acetyltransferase in neocortex and hippocampus in patients (3, 8), including both early and late onset forms of AD (e.g., see ref. 9); (*ii*) basal forebrain neurons, which provide the majority of cholinergic innervation of neocortex and hippocampus, are reduced in number in AD (10, 11); (*iii*) correlation of choline acetyltransferase levels (3, 8) and numbers of basal forebrain neurons (12, 13) with the severity of dementia; and (*iv*) lesions of basal forebrain neurons and pharmacological blockade of mAChR impair cognition in animals (14, 15) and humans (16, 17). That ACh plays a necessary role in learning and memory and that it is sufficient to restore these functions in lesioned animals has been recently demonstrated using cholinergic-specific lesioning methods and genetically modified grafts (15, 18). Moreover, the hypothesis has been further tested in humans by recent clinical therapeutic trials with cholinomimetics. Tacrine, an acetylcholinesterase inhibitor, yields dose-related significant improvements in several measures of cognitive performance and quality of life (19, 20), substantiating a cholinergic role in the pathophysiology of the disease. Yet the overall clinical benefits of this drug are disappointing and may be related to its indirect mechanism of action, which depends on the synthesis, storage, and release of ACh by surviving cholinergic neurons. In addition, side effects resulting from the non-specific activation of cholinergic synapses throughout the body limit the tolerability of the drug and may prevent optimum drug levels from reaching the failing synapses subserving memory and other higher brain functions. However, new insights into the molecular basis for cholinergic neurotransmission, including differential expression of mAChR subtypes at various synapses in memory circuits and surprising findings about how some of these molecules are altered in the disease, together with the development of more specific drugs targeted to receptor subtypes, offer the possibility of improved therapeutic strategies for AD.

The Molecular Diversity of Muscarinic Receptors

Cholinergic neurotransmission is mediated by two classes of receptors, the G-protein coupled muscarinic family and the

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Abbreviations: ACh, acetylcholine; AD, Alzheimer disease; mAChR, muscarinic acetylcholine receptors.

ligand-gated ion channel nicotinic family. Most studies have focused on mAChR subtypes because this family has more established roles in central cholinergic transmission and functions such as learning and memory (3, 21). The molecular diversity of mAChRs became evident with cloning of a family of five genes, m1–m5, encoding highly related but distinct receptor subtypes (22, 23). The lowercase letters "m1–m5" are used to designate the five genes and their products (mRNA and protein), whereas uppercase letters "M1–M4" refer to mAChR subtypes identified in tissues using conventional pharmacological methods, such as differences in binding affinities for various compounds. Each mAChR consists of a single protein, which when stimulated by agonists such as ACh, activate GTP-binding proteins (G-protein) and evoke typically slow, modulatory second messenger responses (24, 25). In transfected cell lines individually expressing each gene, the subtypes differentially couple to intracellular G-proteins and modulate various signaling systems, including phospholipase C, phospholipase D, adenylyl cyclase, nitric oxide, and many ion channels (25, 26). Interestingly, the m1, m3, and m5 receptors also selectively influence the processing of the amyloid precursor protein, such that receptor activation increases the secretion of *non*-amyloidogenic peptides (27). In addition, m1 stimulation dephosphorylates tau in PC12 cells, suggesting that receptor subtypes could potentially alter the hyperphosphorylation of tau proteins and neurofibrillary pathology in AD (28). The heterogeneity of receptors and effectors suggests that the responsiveness of any cell or tissue to ACh will in part be dictated by the subtype(s) expressed.

While a great diversity of behavioral, physiological, and biochemical effects mediated by mAChR have been identified in brain, the identities of the molecular subtypes responsible for any given neural function remain elusive. The complex pharmacology of the mAChR subtypes, together with the lack of drugs having molecular specificity, has made it difficult, if not impossible, to determine the individual roles of m1–m5 receptors in brain. For example, pirenzepine is used to operationally define pharmacological "M1" receptors, although this antagonist has less than a 10-fold difference in affinity between m1 and m4 receptor proteins (29). Similarly, the "M2" pharmacological class is usually defined by AF-DX 384 or related antagonists, compounds that have virtually identical affinities for m2 and m4 receptor proteins (29). In addition, multiple subtypes are undoubtedly involved in various cholinergic responses, whether at a behavioral or cellular level. For example, performance on memory tests in animals is sensitive to a variety of drugs with preference for either M1 (30–34) or M2 receptors (35), suggesting that multiple subtypes are involved. Furthermore, the diversity of muscarinic effects and the presence of each of the molecular subtypes in hippocampus alone suggests that each of the receptors play special roles in memory and other functions involving this structure.

A Diversity of Muscarinic Receptor Actions in Hippocampus

The mAChR subtypes mediate a diversity of pre- *and* postsynaptic actions in hippocampus. Presynaptic mAChRs depress inhibitory and excitatory responses in hippocampus (36–38), with some evidence that different subtypes inhibit release of glutamate, aspartate, γ-aminobutyric acid, and ACh (39, 40). Autoreceptors that inhibit ACh release in hippocampus have been described variously as M2 (39, 41), M2-cardiac like (42), M2-noncardiac like (43), and as M4 (44). The identity of this subtype could be important therapeutically in AD, since antagonists at this site might enhance ACh release from surviving cholinergic terminals (41, 45). Physiological studies also suggest that mAChR are precisely localized at distinct excitatory synapses in hippocampus, where they profoundly influence neurotransmission. Glutamatergic systems are responsible for the majority of excitatory transmission in brain, including the excitatory feed-forward synapses that are the backbone of hippocampal circuitry, i.e., the "tri-synaptic pathway," and are critical for memory and other hippocampal functions. ACh depresses evoked responses at each excitatory synapse. For instance, stimulation of a presynaptic mAChR depresses excitatory transmission at Schaffer collateral synapses in CA1 and at mossy fiber synapses in CA3 (36, 37, 46, 47). Postsynaptic mAChR subtypes also modulate excitatory synaptic neurotransmission in hippocampus (37). An example of this modulation in the CA1 region is enhanced responsiveness of *N*-methyl-D-aspartate receptors by activation of M1 receptors (48). The perforant pathway is also modulated by an mAChR selectively localized in the middle third of the dentate gyrus (49). Thus, although a diversity of pre- and postsynaptic ACh actions in memory-related circuits have been described, which exhibit a high degree of spatial- and subtype-selectivity, determination of the precise identities of the molecular subtypes has not been possible using conventional pharmacological approaches. To gain further insights into the roles of mAChR family, molecular and immunological approaches have been used to study the expression and regulation of the m1–m5 subtypes.

Localization of mAChR Gene Products in Brain

Identification of the mAChR subtypes in brain has been accomplished using *in situ* hybridization to localize the mRNAs (50, 51) and highly selective antibodies to directly quantify (52, 53) and localize the proteins (52). Surprisingly, all of the subtypes appear to be present in brain, albeit in different distributions and relative abundance, as summarized in Table 1. Quantitative immunoprecipitation studies performed by independent laboratories using different antibodies have shown close agreement in the composition of subtypes in

Table 1. Localization of mAChR subtypes in brain

Molecular subtype	Regional abundance	Cellular localization	Synaptic localization
m1	Abundant in forebrain (neocortex, hippocampus neostriatum)	Pyramidal neurons Striatal spiny neurons	Post- ≫ Presynaptic
m2	Moderately abundant throughout brain	Cholinergic neurons, nonpyramidal neurons in cortex and hippocampus	Pre- ≫ Postsynaptic
m3	Low levels throughout brain	Neuronal	?
m4	Abundant in neostriatum, moderate levels in hippocampus and cortex	Striatal spiny neurons Associational and commissural hippocampal projections	Pre- and postsynaptic
m5	Low levels in hippocampus, substantia nigra	Pyramidal neurons, substantia nigra pars compacta, microglia	?

?, Unknown.

various regions of rat (52, 53) and human brain (54). In the forebrain regions of interest for AD, the m1, m2, and m4 proteins are the most abundant subtypes. For example, in hippocampus and several regions of neocortex in human brain, m1 ranges from 35–60% of all mAChR binding sites, whereas m2 and m4 each account for about 15–25% of receptors in the same areas (54). In contrast, m2 is the predominant subtype in the basal forebrain, and m4 is the most abundant mAChR in the caudate and putamen. These findings suggest that m2 may play a role as autoreceptor on cholinergic neurons (see below), whereas m4 may play an important role in motor control and perhaps motor learning. The m3 and m5 receptors are expressed only at very low levels in brain.

Immunocytochemical methods have enabled high resolution localization of the mAChR family of proteins. Light microscopic mapping studies reveal that the proteins (52, 55–57), like the mRNAs (50, 51), are differentially expressed in brain. In fact, all five receptor mRNAs and at least four of the encoded proteins are present in different populations of forebrain neurons in the rat. As shown in Fig. 1, m1–m4 receptors are all expressed in medial temporal lobe structures in non-human primates, with substantial differences in the regional and laminar patterns of immunoreactivity for each subtype. This finding suggests that the receptor proteins are differentially expressed by various neuronal populations and/or differentially transported to pre- and post-synaptic locations. However, as yet there is only limited information available about the precise synaptic distributions of the subtypes.

In neocortical regions and hippocampus, m1 receptor is expressed in virtually all pyramidal neurons, where it is localized in somatodendritic regions. By immunoelectron microscopy, m1 immunoreactivity has been found to be primarily postsynaptic, and quite specifically enriched at certain synapses (Fig. 2). Although ACh is likely to be released at some

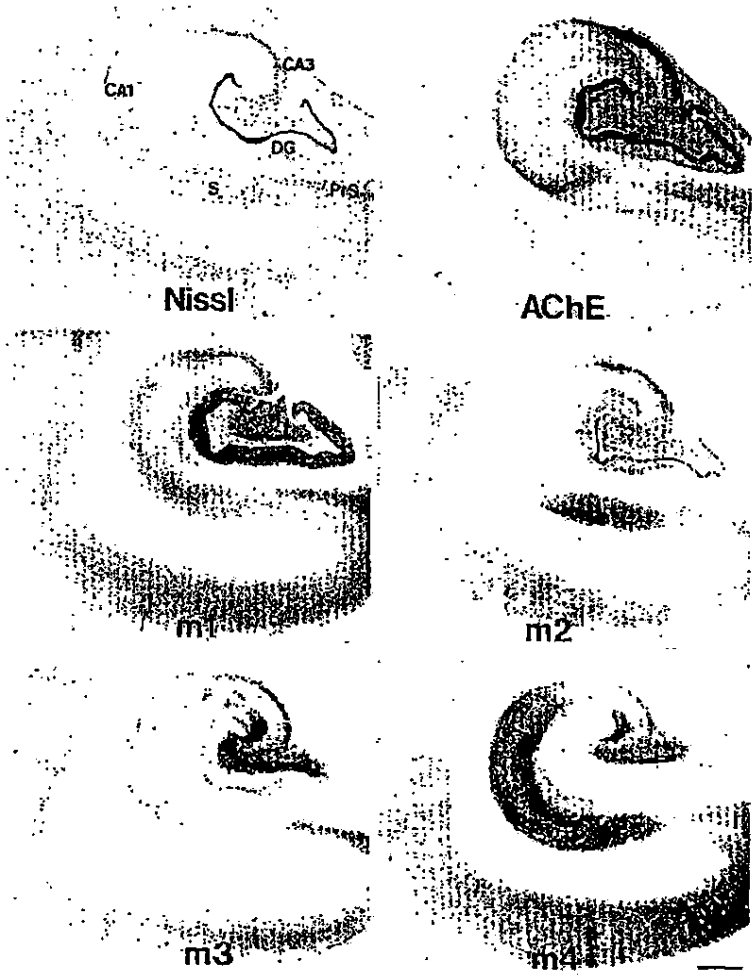

FIG. 1. Immunocytochemical localization of m1–m4 mAChR subtypes in the medial temporal lobe of a non-human primate. Adjacent sections processed for Nissl stain to show the cytoarchitecture and acetylcholinesterase (AChE) histochemistry are shown for comparison. Note the regional and laminar differences in the distributions of the receptors, as well as the highly complementary patterns of expression. (Bar = 1.0 mm.) CA1 and CA3, fields of Ammon's horn; DG, dentate gyrus; PrS, presubiculum; S, subiculum.

of these synapses, no studies have identified the cholinergic terminals directly together with the receptor subtypes. However, many m1-positive synapses appear to receive additional innervation from terminals containing excitatory amino acids (58). As described above, cholinergic modulation of glutamatergic synapses is well established and m1 at such synapses may provide part of the anatomical and molecular basis for this interaction, e.g., as the postsynaptic M1-like receptor underlying the cholinergic potentiation of glutamate *N*-methyl-D-aspartate receptor-mediated neurotransmission (48). Given the failure of glutamatergic synapses in AD, drugs acting at these postsynaptic m1 receptors might augment these crucial memory circuits in the disease as well. The m1 receptor has a similar postsynaptic distribution at excitatory synapses in striatum (59), suggesting that this subtype may play a general role in cholinergic modulation of glutamatergic transmission.

The m2 mAChR subtype has also been of considerable interest for AD, with the assumption that this molecular subtype is a presynaptic autoreceptor that inhibits ACh release (45). For drug development in AD, an antagonist acting at such a receptor would be predicted to increase ACh release from surviving cholinergic terminals. In addition, the effectiveness of subtype-*non*selective agonists or cholinomimetics such as tacrine and other cholinesterase inhibitors might be diminished if the presynaptic autoreceptors are stimulated. While some pharmacological evidence has accumulated for the role of m2 as the cholinergic autoreceptor in cortex and hippocampus (45), this issue has been controversial. Recent molecular and immunocytochemical approaches have provided the first direct assessment of the cellular and synaptic localization of m2 in animal and human brain. In the basal forebrain, the m2 subtype is expressed at high levels in the cholinergic neurons, but it is also present in the admixed populations of neurons that are noncholinergic and which also project to cortex and hippocampus (56). In fact, lesions of the cholinergic neurons that spare the noncholinergic neurons have little apparent effect on m2 receptor expression, indicating that most m2 receptors in this region are located on noncholinergic structures (56). In neocortex and hippocampus, m2 receptors are found in discrete lamina in the neuropil, as well as in certain populations of nonpyramidal neurons (52, 57). As shown in Fig. 2, electron microscopic analysis reveals that many m2 receptors in the hippocampus are present in axons and axon terminals. The m2 receptor is also presynaptic in other regions of the brain, including neocortex (58), basal forebrain (56), and striatum (59). Although ACh may be contained within some of these terminals, many terminals have morphological features suggesting that other transmitters may be contained within. In neocortex and hippocampus most of the presynaptic m2 receptors are probably derived from noncholinergic neurons intrinsic to the cortex and hippocampus (57), because virtually complete lesions of the cholinergic projection neurons have little effect on the abundance or distribution of m2 receptors in the terminal fields. However, the m2 positive terminals in striatum (59), and perhaps a minority of those in cortex and hippocampus, are cholinergic, where this subtype is believed to be an autoreceptor.

Much less is known about the precise localization of the other mAChR in forebrain circuits relevant to memory and cognition. The m3 receptors are present in various neuronal

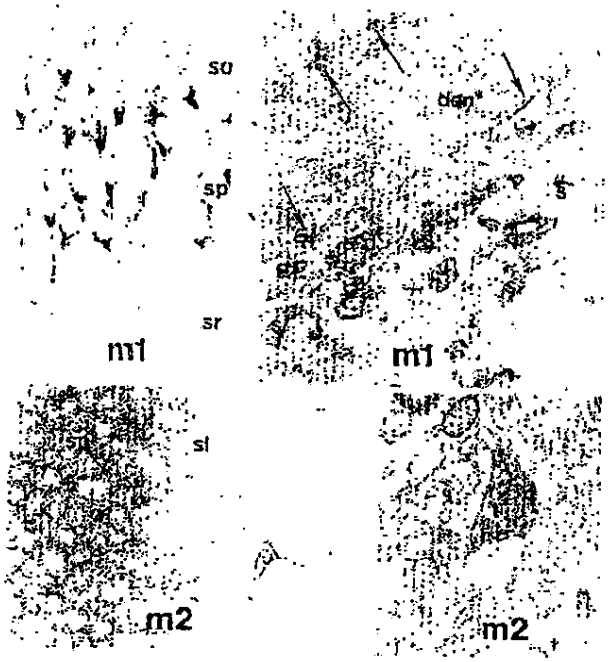

FIG. 2. Light and electron microscopic localization of m1 and m2 immunoreactivity in rat hippocampus. (*Upper Left*) A light photomicrograph showing m1 immunoreactivity in pyramidal neurons in CA1. There is also abundant immunoreactivity in the neuropil in the stratum oriens (so) and stratum radiatum (sr). (*Upper Right*) The stratum radiatum at the electron microscopic level, with m1 immunoreactivity primarily at postsynaptic sites (arrows) along the membrane of a dendrite (den*) of a pyramidal neuron, and with much of the neuropil consisting of immunoreactive dendritic spines (s). In contrast, m2 is primarily localized in nonpyramidal neurons in hippocampus (*Lower Left*). An isolated neuron with several dendrites is seen in stratum lucidum (sl). Also note the dense neuropil immunoreactivity surrounding neurons in the stratum pyramidale (sp). At the electron microscopic level (*Lower Right*), much of the m2 immunoreactivity is presynaptically located within axon terminals (t*).

populations throughout the brain (55). The m4 subtype is fairly abundant in cortex and hippocampus, although it is most enriched in striatum. At the light microscopic level m4 receptors, like m2, appear mostly in the neuropil (57). Although no ultrastructural information has been reported for m4, this subtype is present in several key pathways, including the corpus callosum, hippocampal commissure, and fimbria–fornix. This pattern of localization, together with the laminar distribution of m4, suggests that these receptors may be presynaptically located on associational and commissural projection pathways of the hippocampus. If so, this receptor might be important in the regulation of glutamate release. Finally, m5 is the only receptor protein that has yet to be localized by immunocytochemistry, although the mRNA has been reported in hippocampal pyramidal neurons, dopamine-containing neurons in the substantia nigra, and few other regions (51, 60).

Muscarinic Receptor Subtypes in AD

The ultimate application of basic advances in the molecular neurobiology of mAChR to human conditions, such as AD, depends on knowledge about the expression of the mAChR family in human brain and possible alterations in the receptors by the disease process. Pharmacologically defined binding sites have been analyzed in detail in control and in AD postmortem brain (for review see ref. 61). It has been suggested that "postsynaptic" receptors are largely unchanged in number in AD (45, 57) but may not be functional (62). There has been less consensus regarding "presynaptic" receptors, but some studies have found these are reduced in AD (45). However, because the ligands used in these studies do not have the specificity necessary to distinguish among the individual mAChR proteins or to spatially resolve their pre- and postsynaptic locations, it is difficult to interpret these earlier findings vis a vis the molecular subtypes.

Only recently has any information been obtained regarding the molecular subtypes in AD. A solution hybridization study revealed a significant decrease of m1 mRNA in temporal cortex of six AD patients, with no change in the levels of m2, m3, or m4 (63). No changes in any subtypes were found in other brain regions tested. This finding is at odds with one other study of the mAChR mRNAs, which described an almost 3-fold increase in m1 mRNA in temporal cortex using *in situ* hybridization (64). A recent immunoprecipitation study has provided the first direct analysis of the family of receptor proteins in AD, with assay of several brain regions from 13 AD cases and 11 age-matched controls (54). The results yielded several surprising findings. First, the m1 protein was decreased throughout cortex and hippocampus despite unchanged levels of the M1 binding sites in the same tissues. This finding is in conflict with current dogma, since it suggests that m1, the predominant postsynaptic receptor in cholinergic terminal fields, may be reduced in diseased brain, perhaps as a result of shrinkage or degeneration of pyramidal neurons and their dendrites and spines. Alternatively, the receptor may lose the epitopes recognized by antibodies yet retain ligand binding ability. In addition, there were marked increases in m4 receptors in AD, which occurred only in cortical regions and hippocampus and not in putamen. Because the ligand binding preferences of m1 and m4 overlap (both are "M1"-like), the opposing directions of change in the levels of these two receptors could also reasonably account for the findings of previous studies in which "M1" receptor binding sites were unchanged. Although the cellular basis for the increase in m4 is presently unknown, this subtype might be an interesting target for novel cholinergic therapies. Other subtype changes of note in AD brain include a decrease in the levels of m2 receptor protein. As discussed above, because only a minority of the m2 receptors are present in cholinergic terminals, the reduced levels of this subtype probably reflect changes in other neuron populations, which are intrinsic to the neocortex and hippocampus.

Conclusions

Impaired neurotransmission at muscarinic cholinergic synapses may contribute to the devastating loss of memory and other cognitive abilities in AD. Identification of a family of five mAChR genes encoding highly related receptor subtypes with markedly different cellular and synaptic distributions in brain, raises the exciting possibility that individual receptors may be targets for improved therapies. Presently used cholinergic compounds suffer from a lack of subtype-selectivity and potency, which favor negative peripheral side effects and may limit cognitive effects because of weak and/or opposing actions in brain. In contrast, the high levels and selective expression of several of the molecular subtypes, including m1, m2, and m4, in memory-related forebrain circuits, provides an opportunity for "magic bullet" therapies to be targeted to precise pre- and postsynaptic sites. However, the complexity of cholinergic transmission, together with alterations in these receptors in AD brain, makes predictions about the behavioral and therapeutic relevance of these receptors uncertain. Progress in the development of many selective compounds will likely clarify the ultimate therapeutic implications of the mAChR family for AD in the near future.

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Cover photograph: Window embrasure from the National Academy of Sciences building featuring Baron von Friedrich Heinrich Alexander Humboldt, John Dalton, Chevalier de Jean Baptiste Pierre Antoine de Monet de Lamarck, James Watt, Benjamin Franklin, and Christian Huygens.

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PROGRESS REPORT ON ALZHEIMER DISEASE: Volume III

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service National Institutes of Health

Introduction

Demography

By most estimates, Alzheimer disease is the cause of serious confusion and forgetfulness in some 2.5 million American adults. This is five times the estimate that appeared in the literature 10 years ago when the National Institute on Aging (NIA) began its first studies in this area. Are there so many more victims now than then? Is there an Alzheimer epidemic?

Because aging is the principal risk factor associated with Alzheimer disease, the number of Alzheimer patients is growing at least as fast as the U.S. older population. But these latest estimates stand for more than just growing numbers of older people. They reflect a more sophisticated, if not yet perfected, approach to diagnosis; better training of health care professionals regarding the problems of old age; and the general public's greater awareness of Alzheimer disease and its symptoms.

According to NIA-supported experts at Harvard University, our current estimates of the number of people with Alzheimer disease may still be too conservative. Dr. Denis Evans and his colleagues at the East Boston Neighborhood Health Center have examined the residents of that Boston community and have found that more than 11 percent of the population over age 65 may be suffering from Alzheimer disease. This is double the frequently stated estimates.

When this study has been completed and verified by other studies, and diagnostic capabilities are further refined, we may be surprised to learn how many people are suffering from Alzheimer disease and are being quietly cared for by relatives and friends.

The Cost of Alzheimer Disease

According to the Alzheimer's Disease and Related Disorders Association, $35 billion was spent last year on the care of Alzheimer patients. This includes the costs of nursing home and other long-term medical care, but doesn't begin to account for the emotional and social costs of the disease.

NIA health economist Dr. William Cartwright has tallied some of the specific costs of Alzheimer disease, suggesting that $35 billion is just the tip of the iceberg. Dr. Cartwright and his colleagues, Dr. Lien-Fu Huang of Howard University in Washington, D.C., and Dr. Teh-wei Hu of Pennsylvania State University in State College, estimate

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that the special services required by dementia patients might cost more than $38 billion, with another $39 billion for what the investigators call indirect costs.

The investigators looked at how much money is spent on longer hospital stays, increased need for drugs, greater demands on staff time in nursing homes, special social services and other needs. A large portion of the cost of dementia—$27 billion—reflects the value of the time spent by relatives who care for Alzheimer patients at home.

This is the first time that anyone has calculated the costs of dementia. It is also the first time that anyone has put a value on the so-called "incalculable costs" of the disease. The $39 billion for indirect costs represents the investigators' estimate of the cost of relatives visiting patients in nursing homes, transporting patients for needed medical services, and premature death due to dementia. Dr. Cartwright cautions that these estimates may seem conservative because they include only the extra costs of medical care, rather than all the costs of medical care, for dementia patients.

Diagnosis

There is no single medical test that can diagnose Alzheimer disease. The early symptoms of Alzheimer disease—forgetfulness, confusion, changes in mood and behavior—are also symptoms of a large number of other conditions. As a result, physicians who suspect Alzheimer disease use a variety of tests, including medical history, clinical examination, blood and other laboratory tests, psychological tests and radiologic scans. The diagnosis of Alzheimer disease is made only after all other possibilities have been excluded.

Nonetheless, we are better able to diagnose Alzheimer disease today than ever before. Physicians are more attuned to the various disorders that can mimic Alzheimer disease, and are more likely to detect them during medical examinations. Newer psychological tests have begun to focus more closely on the early and progressive signs of Alzheimer disease. And now, recent developments in radiology have provided diagnostic tools that can better visualize the working human brain.

At the Massachusetts General Hospital in Boston, NIA grantee Dr. John Growdon and his colleagues are using magnetic resonance imaging (MRI), a recently developed diagnostic tool, to examine patients with Alzheimer disease and other forms of dementia. For an MRI scan, the patient is positioned inside a magnetic field crossed by radio frequency waves. The magnetic field causes the body's abundant supply of hydrogen atoms (which are positively charged) to come into alignment; the radio waves then deflect them out of alignment. When the waves are turned off, computers measure the energy emitted as the atoms realign within the magnetic field. By a complex system of calculations, information is then generated on the concentration of matter as well as certain physical and chemical properties.

The technique is safe and painless. In some cases, MRI brain scans may even be superior to CT scans in that the images are crisper, they differentiate between white and grey matter and they can visualize deep areas of the brain not seen in CT scans. Because MRI can more accurately pinpoint tumors and other intracranial disorders, the scans are extremely useful to physicians in diagnosing patients' problems.

Dr. Growdon and his colleagues have found that the unique ability of MRI to distinguish the brain's white and grey matter allows it to pick up abnormalities that may indicate early multi-infarct dementia, or MID (dementia caused by a series of small strokes). MID is the second leading cause of dementia in older people and is frequently

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Whether MRI will be a valuable diagnostic tool for Alzheimer disease is difficult to predict since this technology is still in its infancy. In the meantime, however, Dr. Growdon and his colleagues are excited by their findings of white matter lesions in people who show no other sign of disease. If this is, indeed, an early indication of multi-infarct disease, diet and lifestyle changes may prevent further damage.

As this research progresses, Dr. Growdon will be looking at those areas of the brain known to be affected in Alzheimer disease to see if changes in brain structure can be linked to changes in function as the disease progresses.

A Blood Test for Alzheimer Disease?

While MRI might eventually prove useful in differential diagnosis, a number of studies are holding out the hope of a simple diagnostic test for Alzheimer disease. Anatomically, Alzheimer disease is characterized by neurofibrillary tangles and neuritic plaques, as well as by deposits of amyloid fibers in the brain's blood supply. Dr. George Glenner at the University of California, San Diego has isolated and analyzed the amyloid fibers circulating in the bloodstream of Alzheimer patients and found a unique protein not seen in healthy individuals. Interestingly, the same protein appears in the amyloid deposits of Down syndrome, representing a link between Alzheimer and Down syndrome. With NIA support, Dr. Glenner next plans to use monoclonal antibodies to take a closer look at this unique protein with the hope of isolating its precursor.

This is one of several studies looking at proteins that can be linked to what takes place in an Alzheimer patient's brain as nerve cells begin to die. In studies at the Albert Einstein College of Medicine in the Bronx, New York, scientists have discovered an abnormal protein which they say is found only in the brains of Alzheimer patients, and only in those parts of the brain that are most severely affected by the disease. At Brigham and Women's Hospital in Boston, Massachusetts and the New York State Institute for Basic Research in Staten Island, other scientists have found that a normal protein is somehow altered during the course of Alzheimer disease, and speculate that this may play a role in the death of nerve cells. If scientists can find these same proteins or related proteins in the bloodstream or in some other body fluid, we may soon have a simple diagnostic test for Alzheimer disease.

Biological Mechanisms

In a series of investigations supported by the NIA, Drs. Charles Marotta and Elizabeth Sajdel-Sulkowska of Harvard Medical School and McLean Hospital in Belmont, Massachusetts have found a biochemical abnormality that impedes production of new protein in Alzheimer brain tissue.

For some time we have known that the most serious symptoms of the disease are closely correlated with the intracellular accumulation of abnormal protein structures called neurofibrillary tangles. But scientists have always been puzzled as to how these pathological changes relate to normal cell function.

Drs. Marotta and Sajdel-Sulkowska compared autopsied tissue from the cortex of six Alzheimer patients with that from four patients of the same ages who died of other causes. In the diseased brains, the investigators found massive accumulations of neurofibrillary tangles and little more than half the normal amounts of ribonucleic acid (RNA). RNA is a key chemical in the production of protein, a process that is critical to the life of the cell. A closer look linked the lower levels of RNA to the presence of increased ribonuclease activity. In the healthy brain, ribonuclease breaks down RNA, but its activity is closely controlled by an inhibitor protein. In the Alzheimer brain, Drs. Marotta and Sajdel-Sulkowska found that ribonuclease activity was higher, resulting in more destruction of vitally important RNA which results in less protein synthesis.

This work is an extension of earlier studies in which Dr. Marotta and his colleagues first showed that messenger RNA can be removed from the postmortem brain for scientific study. Until Dr. Marotta's pioneering work, it had been assumed that large molecules such as RNA and deoxyribonucleic acid (DNA) were broken down very quickly after death. This might have been the case using more traditional chemical methods for storing brain tissue. Formalin, the chemical used to fix brain tissue after death, destroys enzymes and other fragile biochemical substances in the autopsied brain. But most of these problems are alleviated when autopsied brain tissue is frozen and later thawed for study. According to Dr. Marotta, if the brain has been frozen and stored, RNA will synthesize protein as if in a living brain, even years after death.

As the investigators are quick to point out, their work on autopsied brain tissue has already been corroborated in living patients. Using positron emission tomography, a noninvasive technique which

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monitors metabolic activity in living brain tissue, a group of French scientists has found a 65 percent drop in brain protein synthesis in patients in the later stages of Alzheimer disease.

It is not possible to state if this change marks the onset of Alzheimer disease, or if it is just one of a series of events that takes place as brain cells begin to die. If, as the investigators speculate, decreased protein synthesis results in the chemical changes and brain cell death characteristic of Alzheimer disease, then we might be one step closer to finding out what causes this debilitating illness.

Changes in Brain Chemistry

In addition to the accumulation of neurofibrillary tangles in the Alzheimer brain, another hallmark of the disease is a disruption of one of the brain's chemical messenger systems, the cholinergic system. With support from the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS), Dr. Joseph Coyle and his colleagues at The Johns Hopkins University in Baltimore, Maryland, have shown that one message pathway in the cholinergic system that deteriorates in the brains of Alzheimer patients also is destroyed in a rat model. This discovery might help explain why the brain's cortex and hippocampus, which lie at the end of this pathway, accumulate tangles and plaques, and why Alzheimer patients lose short-term memory governed by the cholinergic system but retain the long-term memory maintained by other chemical messenger systems.

Dr. Coyle has simulated cholinergic system deterioration in rats using a chemical that damages only cholinergic neurons. His study suggests that memory loss in the rat model of Alzheimer disease may be reversible: animals with induced cholinergic damage eventually regained some of their short-term memory.

In yet another study, NIA/NINCDS grantee Dr. Marek-Marsel Mesulam and his colleagues at Beth Israel Hospital in Boston, Massachusetts, have identified a distinct group of cholinergic neurons extending from the basal forebrain along cholinergic pathways and linking selected neurons in the basal forebrain with particular regions in the cortex. The investigators previously had demonstrated that changes in such cell groups can trigger the kind of brain dysfunction associated with Alzheimer disease. Studies of the newly discovered cell group show that these cells influence a region of the brain that transmits visual and other sensory information.

The Search for a Cause

The theories of what causes Alzheimer disease have matured in a rich atmosphere of research focused on the neuroscience of aging. The basic theories have not changed. Scientists still think that genetic factors; immunologic changes; unconventional virus-like agents; and environmental factors may all play a role in the development of Alzheimer disease. Nonetheless, more basic answers are being sought as to what causes the disease. NIA-supported scientists are examining the fundamental processes that underlie changes that take place in the brains of patients with Alzheimer disease.

At the Burke Rehabilitation Center in White Plains, New York, NIA grantee Dr. John Blass and his colleagues are doing research on enzyme activity, calcium transport, glucose metabolism and protein synthesis in an effort to reveal the events that precipitate neurotransmitter changes and even cell death in Alzheimer disease. Their search has led them to look at tissues, fluids, and cells outside the brain. Based on their results, they have speculated that Alzheimer disease is a systemic metabolic disease, not simply a neurologic disorder.

For some time, scientists have known that there is a drop in overall glucose metabolism in the brains of Alzheimer patients; in other words, the Alzheimer brain uses less energy. Dr. Blass and his colleagues looked at glucose metabolism in tissues taken from Alzheimer patients and found that there was an inherent abnormality in the cells' ability to break down glucose into energy. They also found a defect in calcium transport at the cellular level.

According to Dr. Blass, Alzheimer disease may be a neurologic disease with systemic manifestations or a systemic disease that shows itself primarily in terms of brain changes. This realization may alter the approach to studies of how the disease develops and how it can be diagnosed and treated.

Alzheimer and Pick Disease: A Common Cause?

Other research continues to alter our understanding of Alzheimer disease. At Harvard University, NIA grantee Dr. Dennis Selkoe has found a surprising similarity between Alzheimer disease and Pick disease, a rare, rapidly progressive dementia.

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Alzheimer and Pick diseases have always been presumed to be distinct neurological disorders. Although victims of both diseases suffer disabling dementia, neurofibrillary tangles and neuritic plaques are not found in the brains of individuals who had Pick disease. Only an autopsy can distinguish between the two diseases.

Earlier studies by other investigators had shown that both Alzheimer and Pick diseases affect the same parts of the brain. Research had even speculated that although the lesions of Pick and Alzheimer are different, they might be composed of the same abnormal proteins called paired helical filaments (PHF). Following these leads, Dr. Selkoe used a technique developed in his laboratory to study nerve cells from the brains of Alzheimer and Pick victims. He found that the PHF's once thought to be unique to Alzheimer disease were indeed located in the brain cells of Pick patients.

Dr. Selkoe speculates that Pick disease may be a variant of Alzheimer disease and that the two may share a common cause. This opens up a new field of investigation.

The Aluminum Story

When scientists first looked at the brains of Alzheimer patients, they found excessive levels of aluminum concentrated in dying brain cells. These findings led to fearful speculation by the public that Alzheimer disease could result from ingesting aluminum or using household products containing aluminum. Fortunately, epidemiologists have ruled out such a simple answer to what causes the disease, and our approach to studies of environmental risk factors has become much more sophisticated.

NIA grantee Dr. Bernardino Ghetti has confirmed that when the central nervous system is exposed to aluminum over long periods of time, the result is neurofibrillary degeneration and ultimately cell death. In his work at Indiana University School of Medicine in Indianapolis, Dr. Ghetti is beginning to elucidate the mechanisms by which aluminum causes nerve cell damage. He has found that aluminum binds to the protein calmodulin, impairing its ability to regulate calcium levels in the cell. Calcium is similar in structure to aluminum and is vital to the life of the cell but toxic in large quantities. According to Dr. Ghetti, the key to aluminum's deadly effect on nerve cells may be this breakdown of calcium metabolism at the cellular level.

The Role of Infectious Agents

Since the late 1960's, scientists have been exploring the role of infectious agents in several dementing disorders. Today, some scientists are still looking to see if such an agent might be implicated in Alzheimer disease. Dr. Stanley Prusiner of the University of California at San Francisco—a grantee of the NINCDS and the NIA—first identified the "prion," which causes some infections that reside within the body for years before producing symptoms, as well as a specific protein that is the major component of the prion. Dr. Prusiner's latest research suggests that the prion protein comes from a larger protein present in both normal and infected cells. In normal cells, the larger protein is completely broken down by enzymes; in infected cells, a defective breakdown leaves the prion intact. According to Dr. Prusiner, this prion aggregates to form a fibrous structure that resembles certain filaments found in the brains of Alzheimer patients. Dr. Prusiner is now trying to determine what might happen to change a normal cell into an infected cell and why it appears that the larger protein behaves differently in infected cells than in normal ones.

Related Research

The hypothesis that an infectious agent is the cause of Alzheimer disease is based in part on the known transmissibility of certain other degenerative brain diseases in both humans and animals.

One animal model is scrapie, a slowly progressive neurologic disease of sheep and goats. Scrapie, which can be transmitted to a variety of animal species including mice, hamsters, and mink, is so named because stricken animals repetitively scrape against fence posts and trees. As the disease progresses, the animals degenerate steadily, become paralyzed, and die.

Scrapie infection is characterized by large fibril structures and neuritic plaques in brain tissue, features strikingly similar to pathologic findings in patients with Alzheimer disease. These plaques are found in many chronic diseases, although none has been detected so far in diseases in which viruses have been proven to be the cause.

Purified samples of scrapie-infected tissue have been shown to transmit disease to healthy laboratory animals. Upon close examination, the prion protein has been found to predominate in the infected tissue.

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At the National Institute of Allergy and Infectious Diseases (NIAID) intramural Rocky Mountain Laboratories in Hamilton, Montana, Dr. Bruce Chesebro and his colleagues have discovered the genetic material for this prion protein in both healthy and scrapie-infected animals, indicating that the protein is probably a component of normal brain tissue.

Dr. Chesebro examined the brains and other organs of healthy and scrapie-infected mice, using a chemical probe designed to detect the messenger ribonucleic acid (mRNA) sequence that corresponds to the prion protein. Messenger RNA is the molecule that transmits the DNA message to the cell's cytoplasm where it directs protein synthesis. The mRNA sequences were found in tissues of both healthy and infected animals. In no case was the mRNA specific for scrapie-infected tissue.

Dr. Chesebro suggests that prions may simply be part of a healthy cell's structure, and some aspect of the infection causes the protein to bind together to form the deposits found in infected animals. In other words, accumulated protein may be the result of scrapie infection rather than the cause.

Dr. Chesebro's work does not explain the nature of the scrapie agent. His findings do make conceivable the theory that very small virus particles may be responsible for scrapie and other slowly developing diseases.

The Genetic Hypothesis

A discussion of possible causes of Alzheimer disease would not be complete without some reference to genetic studies. In an NINCDS-funded study of 3,500 nursing home residents, Dr. Marshall Folstein at The Johns Hopkins University found evidence of an autosomal dominant pattern of inheritance in certain families with high rates of Alzheimer disease. This could mean that each child of a parent with the familial form of Alzheimer disease has a 50 percent chance of developing the disease in later life.

It seems apparent from our research thus far, however, that the majority of Alzheimer victims don't inherit the disease from their parents. Despite evidence of a familial form of Alzheimer disease, it may be far more common for people to inherit a predisposition to the disease. In an NIA-supported study at the Bronx Veterans Administration Medical Center in New York City, Dr. John Breitner has found that Alzheimer patients are much more likely than those without the disease to have a close relative with dementia.

Both of these studies lend support to the position that genetic factors play a significant role in Alzheimer disease.

Risk Factors in Alzheimer Disease

A team of American and Italian investigators recently reported on the largest case-control study of Alzheimer risk factors, completing a 3-year collaborative effort of the Italian National Research Council and the NINCDS. The findings indicated that people whose brothers or sisters have any form of dementia may be 11 times more likely than others to develop Alzheimer disease. The study also provided some support for earlier observations that severe head trauma may be a risk factor for Alzheimer disease, and that babies born to mothers over age 40 may be at greater risk for dementia later in life.

This study, carried out at seven Italian medical centers under the Science and Technology Agreement between Italy and the United States, did not support risk factors suggested by earlier, smaller case-control studies: family history of Down syndrome, previous thyroid disease, exposure to aluminum or other toxins, allergies, previous surgical procedures, habits of smoking or drinking wine, or certain personality traits. The results did suggest that diagnoses of Alzheimer disease may vary greatly according to the patient's socioeconomic status, probably because mental impairment is more noticeable in people whose education or lifestyle reflects a certain degree of intellectual achievement.

In addition to their work on this project, NINCDS intramural investigator Dr. Bruce Schoenberg and his research team have reported on the occurrence of Alzheimer disease among the 24,000 residents of Copiah County, Mississippi. This landmark study measured the incidence and prevalence of various neurological disorders in a racially mixed population. The results demonstrated that the rate of Alzheimer disease is similar among both blacks and whites. Furthermore, twice as many women as men had Alzheimer disease, and the number of Alzheimer patients increased with advancing age, from 1 percent among people age 40 years and older to 7 percent among those 80 years and older. The range of other estimates of Alzheimer disease indicates that more research in different populations may be needed to establish firmly the incidence of the disease. Nonetheless, this study reconfirmed that aging is one of the major risk factors for Alzheimer disease.

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Assessment and Treatment

Several times during the past few years, the media have publicized major improvements in Alzheimer patients given experimental drugs. Each time, a closer examination has revealed that there is still a great deal of work to be done. Scientists now find it necessary to use a variety of tests to evaluate their patients' responses to new drugs, without knowing how reliable the tests results are. A pressing need is for a test that can be used to assess the potential of any drug.

At the Bronx Veterans Administration Medical Center in New York City, NIA grantee Dr. Kenneth Davis has devised a simple test that focuses on the major symptoms of Alzheimer disease and can be used to evaluate patients in all stages of the disease. The test is brief, easy to administer, and can be used anywhere the patient is located. It evaluates such factors as memory, language, mood and behavior; it rates the majority of the items on a five point scale of severity so that subtle changes can be detected.

Dr. Davis and his colleagues originally designed a scale with 40 items. After more than a year of tests, they have pared the list to 21 items they believe are valid and reliable measures of any change in a patient's symptoms of Alzheimer disease.

It is hoped that the Alzheimer Disease Assessment Scale will be sensitive enough to measure the success of any future attempts to treat the disease.

Treatment Approaches

As noted above, patients with Alzheimer disease often have a marked reduction in cells that produce acetylcholine, one of the neurotransmitters that allows nerve fibers to send electrical messages. The loss of these cells may be a cause of some symptoms of the disease. In an effort to increase nerve cell activity associated with acetylcholine, Dr. Richard Mohs and his colleagues at the Mount Sinai School of Medicine in New York City gave 12 patients oral doses of the drug physostigmine, which mimics the action of the neurotransmitter. The tests were conducted in the Mount Sinai General Clinical Research Center (GCRC), one of 78 such centers supported by the National Institutes of Health's Division of Research Resources.

Previous studies at the Mount Sinai GCRC and other medical centers had indicated that intravenous use of the drug temporarily improved the memory of Alzheimer patients. Uniform levels of physostigmine, however, were difficult to maintain due to its short (30-minute) half-life in the bloodstream. Other tests had indicated that a constant level of the drug may be achieved if it is taken orally every 2 hours. The investigators found varying improvements in the memory, sleeping patterns, and behavior of Alzheimer patients during two studies with the drug.

In the first study, designed to find the most effective dose of physostigmine, 10 of the 12 patients significantly improved their scores on the Alzheimer Disease Assessment Scale, the evaluative test described above. But in a followup test that compared a placebo with the physostigmine dose believed to be most effective, only three patients showed significant improvement using physostigmine; four others improved slightly.

According to Dr. Mohs, drugs that increase acetylcholine activity may help some patients with Alzheimer disease, but further research is needed to identify safer, longer-acting drugs.

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Conclusion

The National Institute on Aging hopes to continue to expand its research on Alzheimer disease. Studies will focus on analyzing the protein chemistry of normal and abnormal brain structures; developing monoclonal antibodies for specific brain proteins; looking at brain enzymes, particularly those related to oxidative metabolism and neurotransmitter synthesis; and measuring the effects of toxins, infectious agents and genetic factors on brain degeneration. NIA will continue to support research on ways to make an early and accurate diagnosis of Alzheimer disease, and to use improved diagnosis as the basis for establishing multinational epidemiologic studies. Through a cooperative arrangement with NINCDS, NIA will provide cells from definitely diagnosed Alzheimer cases to investigators studying the molecular biology and genetics of the disease. We also hope to expand research on the behavioral and social aspects of the disease, including how families cope under such tremendous stress.

In 1984, the NIA was authorized by the U.S. Congress to establish Alzheimer Disease Research Centers, 10 of which are currently being funded. The most exciting opportunity for future research on Alzheimer disease rests in the potential of these centers to act as a network for sharing new ideas as well as research results. Already, the center directors are communicating about such matters as joint patient registries; shared data, tissue, and brain banks; and standardized diagnostic criteria. The Alzheimer Disease Research Centers share a common goal: to enhance research on the disease by providing the resources and environment for collaborative studies among many scientists from many different disciplines. The long-term reward may be a way to cure and possibly prevent Alzheimer disease. The immediate payoff will be better care for more of its victims.

Alzheimer Disease Research Center Program

The National Institute on Aging currently funds 10 Alzheimer Disease Research Centers (ADRC's) in a program designed to speed us toward an understanding of what causes the disease and what can be done to treat it. Research topics range from studies of the basic mechanisms of Alzheimer disease to those aimed at managing the symptoms and helping families to cope, with each of the 10 centers having its own unique areas of emphasis.

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A Comparative Study of Galanthamine hydrobromide and Atropine/Neostigmine in Conscious Volunteers

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Eingegangen am 14. Mai 1971

Vergleichende Untersuchungen von Galanthaminhydrobromid und Atropin/Neostigmin bei wachen Versuchspersonen

Zusammenfassung. Bei wachen Versuchspersonen erwies sich Galanthaminhydrobromid als mildes Analeptikum. Es ist in der Lage, die Pulsfrequenz und den Blutdruck ohne Atropin im Normbereich zu erhalten. Die Zahl der Eosinophilen stieg leicht an, blieb jedoch immer noch innerhalb der Norm. Die subjektiven Reaktionen glichen denen nach Gabe einer Anticholinesterase.

Summary. In conscious subjects, galanthamine hydrobromide, has shown itself to be a mild analeptic. It is able to maintain normal heart rates and blood pressure without atropine. Although the eosinophil count rose, it remained within normal limits. Venous blood sugar level was unchanged. The subjective symptoms were similar to those one would expect as a result of the administration of an anticholinesterase drug.

According to many authors the advantages that galanthamine hydrobromide (Nivalin, Pharmachim, Sophia, Bulgaria) might have over atropine-neostigmine are its ability to reverse safely a non-depolarising block without requiring atropine, and its analeptic effect.

An attempt to record EEG changes during general anaesthesia was found quite difficult owing to electrical disturbances as from electro-cautery, and the inability to keep the patient being reversed absolutely still so as not to interference with the EEG. It was, therefore, decided to administer galanthamine hydrobromide (Nivalin) to a group of conscious volunteers and to compare this group with one having received atropine-neostigmine. In this way it was possible to determine whether galanthamine hydrobromide does have any central effect in itself without any complicating factor such as other drugs normally used in general anaesthesia.

In a previous account (by Cozanitis, 1971 [1]), the wheal produced by an intradermal injection of the drug was four times as large as that following a control sterile distilled water injection. In the present study, eosinophils in venous blood before and after anticholinesterase drugs were examined. In addition, heart rate, blood pressure, EEG, blood sugar, and subjective symptoms were recorded.

Method

Two groups of ten healthy volunteers, all female nurses, were examined. In the group receiving galanthamine hydrobromide, the mean age was 27.4 years and mean weight was 58.2 kg. The corresponding group had a mean age of 27.1 years and mean weight of 57.7 kg. The subjects were to have no history of convulsions or asthma and to be free of any cardiac condition. In addition, they were to be of the "not-anxious" type and had not taken any tranquilizers or sleeping tablets for at least ten days prior to the test. In order to limit the dizziness and vomiting resulting from the anticholinesterase drugs, they were to be on an empty stomach for a minimum of four hours. An eight-channel Offner EEG with 23 scalp electrodes, one channel for ECG, was used. The electrode placement followed those of the International 10/20 system. Different montages were used during the 30 min pre-injection period to ensure an adequate evaluation of the normal state. Photic stimulation was used before the injection, after the last increment of the drug, and then at 5 min intervals for 30 min to elicit possible activation effects as seen in Photo-Metrazol activation [3]. Special attention was paid to the state of vigilance of the subject to differentiate the beginning of progressive slow-synchronization (analeptic) effects from that seen in rudimentary form of reduced vigilance. Recordings were classified according to generally accepted grounds [4]. The activation effect was read on a five-point scale: one, accentuation and slight slowing of frontal alpha; two, episodic theta activity; three, paroxysmal theta; four, delta activity; and five, spike and wave formations.

A Riva-Rocci cuff was placed on the left forearm and a no. 20 Viggo needle inserted into the left cubital vein. 2 ml of blood were removed via the needle and placed into an EDTA-coated container, and gently mixed for eosinophil and blood sugar determinations. Blood pressure was measured by auscultation of the Korotkov sounds and heart rate measured by palpation of the radial pulse. This latter measurement could be confirmed by the ECG tracing. Diazepam 10 mg was on hand in case a seizure would result, as was atropine 2 mg in case of severe bradycardia. The subject's normal heart rate and blood pressure, as known to her, were recorded as well as the values one minute before the initial injection of the drug. The vein was kept open by means of a slow drip of normal saline.

When galanthamine hydrobromide was used, 0.5 ml (5 mg) was injected every minute to a total of 2 ml (20 mg). Blood pressure and heart rate were measured at minute intervals and after the last increment of galanthamine, at five minute intervals for a total of 30 min. During this time, EEG and ECG were constantly recorded. The subject was asked to report any strange sensation using 1 or 2 words. In addition, questions as to symptoms were asked by the examiners and answers were to be kept to "yes" or "no", when possible. This was so as not to interfere with the EEG. The same method was used with the group receiving atropine-neostigmine with the exception of administration. Here, atropine 1 mg was given rapidly intravenously in a single dose. Blood pressure and heart rate were measured one and two minutes after administration. It had been decided not to give neostigmine until a definite rise in heart rate was seen. Only then was neostigmine given and then in four increments of 0.5 mg/min. Neostigmine 2.5% was used and given via a tuberculin syringe of 1 ml capacity. At the end of the thirty minutes, 5 ml of blood was removed via the needle and discarded. A second sample of 2 ml was collected for examination of eosinophils and blood sugar. The subject was kept in a semi-prone position until the electrodes were removed. After 20 min she was allowed to get up, first being warned about dizziness. The subject was questioned as to subjective symptoms as nausea, dizziness, vomiting, abdominal sensations, twitching, fasciculations, and respiration. Approximately one hour after the thirty-minute period and only when all symptoms had disappeared, she was sent home via taxi and told to report immediately any bradycardia or ill-effects.

Results

The resting EEG and response to photic stimulation in all twenty subjects were within normal limits. Compared to the pre-injection period the EEG findings were as noted in Table 1:

Table 1. *EEG changes occuring after anticholinesterase drugs*

Change	Galanthamine group	Atropine/ neostigmine group
None	1 subject	9 subjects
Slight	5 subjects	1 subject
Mild	4 subjects	0

In the galanthamine group a slowing effect appeared during the first 2 min after the last increment was given. In most cases, however, it was evident after the third increment, and the effect lasted 5 to 30 min (mean 20 min). A typical "mild" change, two points in the 5-point scale, due to galanthamine is seen in Figs. 1 and 2.

During the pre-injection period five subjects of the galanthamine group showed a normal occipital photic response, whereas all ten of the corresponding group manifested this response. With galanthamine, 7 subjects showed enhancement of the response on photic stimulation. Here, the photic evoked activity was more easily elicited or accentuated immediately after the last increment, and this effect lasted from 5 to 30 min (mean 15 min). In the atropine/neostigmine group (Table 1), one subject showed an accentuated response which lasted for five minutes.

The range of normal heart rate in the galanthamine group was 58 to 84 (mean 74). Five minutes after the last increment it was 60 to 120 (mean 85) and at thirty minutes, 58 to 102 (mean 76), (Fig. 3). Paskov [5], has shown that the intravenous injection of galanthamine resulted in an increased heart rate. With this information in mind, a one-tailed *t*-test was used. All the differences were statistically significant as seen in Table 2.

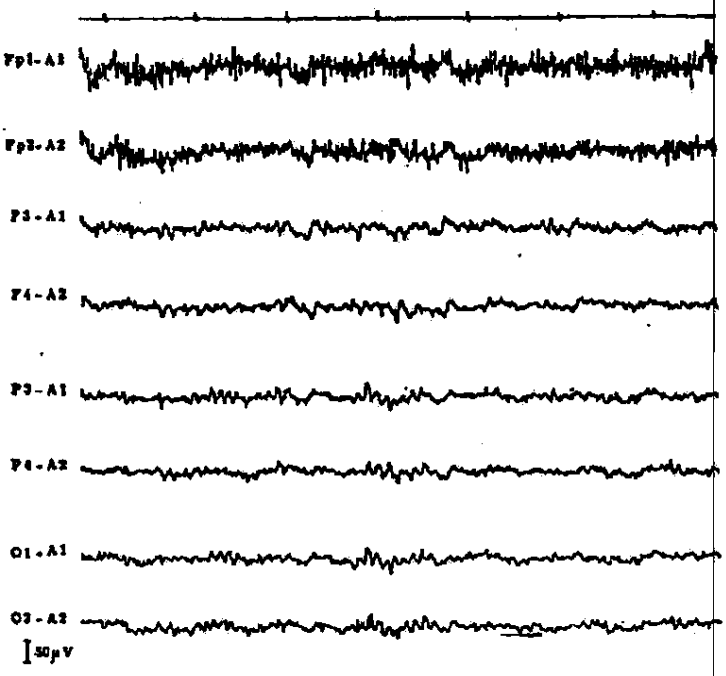

Fig. 1. EEG pattern before galanthamine. (Symbols after Internat. 10/20 system)

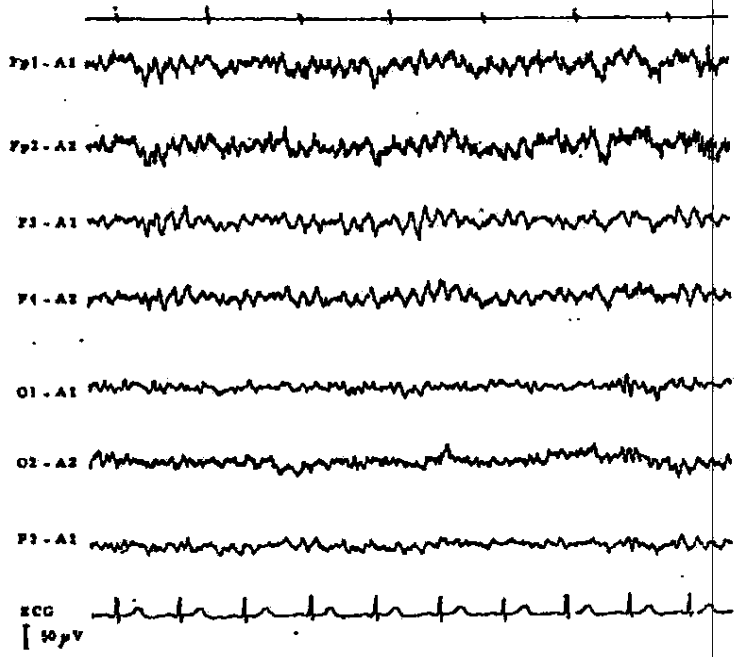

Fig. 2. 5 min 20 sec after last increment of galanthamine. Abundant theta activity in frontal areas of brain

Table 2. *The statistical significance of differences between the means (normal–5 min, 5–30 min, normal–30 min). df=9*

Condition	"t" value	$P<$
Normal to 5 min	3.511	0.005
5–30 min	2.500	0.025
Normal to 30 min	2.042	0.05

The change from Normal to 30 min has presumably no biological significance.

Table 3

Effect of 20 mg Galanthamine Hydrobromide on Eosinophilic Count/mm³

Subject	Before Galanthamine	After Galanthamine
1.	110	176
2.	165	231
3.	11	55
4.	55	99
5.	11	33
6.	22	55
7.	22	66
8.	99	154
9.	0	11
10.	55	88
Mean	65	97

Effect of 1 mg Atropin and 2 mg Neostigmine on Eosinophilic Count/mm³

Subject	Before Atropine-Neostigmine	After Atropine-Neostigmine
1.	66	77
2.	22	55
3.	33	0
4.	66	99
5.	44	55
6.	33	33
7.	121	143
8.	231	297
9.	66	88
10.	22	44
Mean	70	89

In the atropine/neostigmine group, the normal heart rate ranged from 60 to 70 (mean 68). The rapid intravenous injection of atropine 1 mg after 1 min, led to a highly significant increase (range 82 to 124, mean 105), and after 2 min the range was 100 to 136 (mean 120). Five minutes after the last increment of neostigmine, the range was 58 to 92 (mean 72), and at thirty minutes, 58 to 72 (mean 66).

The resting (mean) blood pressure in the galanthamine group was 121/73 mm Hg and in the second group, 115/72 mm Hg. This rose slightly in both groups five minutes after the last increments of galanthamine and neostigmine (Fig. 4). The blood pressure of the galanthamine group then very gradually diminished until at the end of the 30 min recording period, it was approximately at normal levels. In the neostigmine group, it fell slightly below the normal value during the first 15 min and then gradually increased until it was practically normal again at the end of the trial. In both groups, the biggest rise in

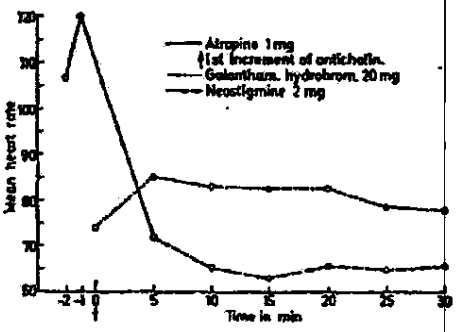

Fig. 3. Comparison of mean heart rate following galanthamine hydrobromide and atropine/neostigmine

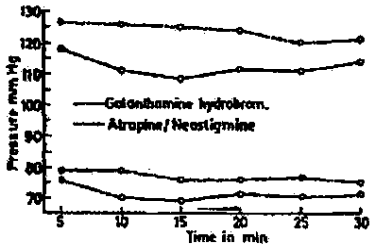

Fig. 4. Comparison of systolic and diastolic changes occuring 30 min after injection

blood pressure was 20 mm Hg. The biggest drop in the galanthamine group was 15 mm Hg and in the group having atropine and neostigmine, 35 mm Hg. Blood sugar remained unchanged in both groups. Eosinophil cells were all counted by the same technician (Table 3). In all ten cases having received galanthamine, this amount rose thirty minutes after the last increment. Using a one-tailed *t*-test, this rise was statistically significant: $t=7.421$, $df=9$, $P<0.0125$. In the neostigmine group, there was an increase in 8 cases, 1 showed a slight decrease and one remained unchanged. Here, a two-tailed *t*-test showed the rise being statistically almost significant: $t=2.325$, $df=9$, $P<0.05$. However, in all cases the final value was well within the normal limit of 400/mm³.

The ECG was monitored with 2 chest leads and, therefore, cannot be considered very accurate from a cardiological viewpoint. However, no obvious changes were noted and atropine did not seem to alter the ECG pattern.

Table 4 lists the various subjective symptoms experienced by the subjects after administration of the anticholinesterase drugs. Nausea, vomiting, and dizziness occurred more in the galanthamine group, and inspite of the 1 mg of atropine given before neostigmine, abdominal sensations were very frequent in that group. Twitching of eyelids was apparent in both groups but fasciculations were experienced only in those having received neostigmine. A most interesting effect was that of "swollen" throat noted by nine subjects of the second group. The effect on breathing was apparently central in the galathamine group and mechanical in those of the neostigmine group.

Table 4. *Subjective Symptoms due to two Anticholinesterase Drugs*

Effect:	Nausea	Vomiting	Abdom. Sensation	Dizziness	Eyelid Twitching	Fasciculat.	Blurred Vision	Warmth	Breathing Affected	Swollen Throat
Galanthamine Hydrobromide	5	6	1	5	6	0	4	5	3	0
Atropine/ Neostigmin	3	0	6	2	7	5	5	1	3	9

Discussion

Generally speaking, an analeptic is a central nervous stimulant. A more precise definition would be: a drug which in suitable amounts will cause numerous progressive somatic, vegetative, psychic effects and even epileptic seizures. In electroencephalography this denotes a slow synchronization effect: spread of spontaneous alpha activity to the anterior areas of the brain, increase in voltage, decrease in frequency, appearance of slower synchronous wave groups and paroxysms, followed by spike and wave complexes. The pre-existing changes will be enhanced and evoked potentials are facilitated as well. In most subjects of the galanthamine group there were changes towards slow synchronization, mostly frontal episodic theta activity. Seven subjects of this group manifested an accentuated response on photic stimulation, as compared to one case belonging to the neostigmine group. According to these EEG findings, galanthamine is an analeptic drug, although mild. Nevertheless, with strong analeptics as Metrazol and Bemegride, the EEG changes are sometimes mild or even absent until a sudden appearance of spike and wave paroxysms with or without clinical seizure. Galanthamine in clinical doses seems unable to provoke an epileptic seizure, and in a current parallel study here, the injection of the drug into known focal epileptics has failed to accentuate focal EEG changes whether absent or evident in the pre-injection period.

The effect on heart rate after galanthamine, on conscious subjects was similar to that noted on anaesthetised patients [1]. The immediate slight bradycardia was followed by an increase returning to normal at approximately 30 min.

Three factors must be borne in mind when comparing the 2 anticholinesterase drugs used in this study. First, the central action of galanthamine, second, the fact that neostigmine, according to Salvini, Frosali, and Pacetti [6], is 20 times more potent, and third, that due to the very slight muscarinic effect of galanthamine, no atropinization is necessary. The subjective symptoms elicit pharmacologically the classical responses seen with physostigmine. This could be better appreciated in the galanthamine group, which as mentioned above, received no atropine. For example, warmth due to circulatory response causing peripheral vasodilatation, and vomiting due to the stimulation of the smooth muscle of the intestine (which in the neostigmine group was not manifested, as atropine blocks the effect of anticholinesterases on smooth muscle). Atropine does not, on the other hand block the effect on striated muscle and, therefore, twitching of the eyelids (stimulation of levator palpebrae) was noted in both groups. Fasciculations were very prominent in the neostigmine subjects due to the high relative potency of the drug. Pharmacologically, this symptom according to Wylie and Churchill-Davidson [8], is due to the accumulation of packets of acetylcholine at the endplate and to the direct depolarizing action of neostigmine on the endplate with antidromic excitation of the remainder of the motor unit.

Blurring of vision occurred in both groups probably due to loss of accommodation by the paralysis of the ciliary muscle. As this muscle is of smooth variety, one would expect to see this effect blocked by atropine.

Atropine 1 mg was not able to suppress the effect of neostigmine on the intestine in 60% of the subjects. This abdominal effect was, after "swollen throat", the most uncomfortable symptom. Nevertheless, the antiemetic of atropine [2] might have been the reason for the absence of vomiting in this same group.

Three members of both groups experienced respiratory symptoms. However, the symptoms were of a different nature in each group. Those having received galanthamine were seen taking deep breaths or sighs and the alae nasae being used. The subjects of the neostigmine group complained of "swollen throat" to the extent that for some minutes swallowing was difficult and at times, impossible. Probably because of this, breathing was found difficult. It might be concluded that as galanthamine does have a central stimulatory effect, and that the symptoms experienced might be due to stimulation of respiratory centres. The neostigmine effect was obviously mechanical in nature. One might, in view of this observation, question whether some of the immediate post-operative complaints of sore throat might not be altogether due to the endotracheal tube.

Eosinophils comprise 1 to 5 percent of the total leukocyte count (40 to 400/mm³), in normal individuals. One of the functions of these cells is to inactivate histamine. A local antigen-antibody reaction or release of histamine brings the eosinophils to the reaction site. By some direct chemical effect, they inactivate the histamine. If the reaction is of mild nature the circulating (transit) cells move to the tissue site from the available pool of eosinophils situated mainly in the bone marrow. If, however, the stimulus is maintained, and the marrow pool becomes depleted, then the maintenance is dependent on increased production by the marrow.

The amount of eosinophils in the venous blood was slightly though significantly increased in the group having received galanthamine. The resulting intradermal reaction appeared in all cases to be short-lived, disappearing after approximately one hour and the number of eosinophils 30 min after the injection was still within the normal limit mentioned by Wetherly-Mein [7].

Acknowledgements. My thanks are again due to my friend Dr. Richard S. J. Clarke, Belfast, for his interest, his help, and his criticisms. (D. A. C.). We are very grateful to Pharmachim, Sophia, Bulgaria, for their generous supply of Nivalin.

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Änderungen der blutdruckregelnden Reflexe bei der Neuroleptanalgesie

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Changes in Blood Pressure Regulating Reflexes under Neuroleptanalgesia.

Summary. The changes in blood pressure caused by enteroceptive reflexes have been examined on mongrel dogs. Traction of the stomach during anaesthesia using Chloralose caused a certain fall in blood pressure, but an increase was observed using Neuroleptanalgesia II. This difference is probably caused by the functional state of the cortex which influences the reflex arc in the spinal cord.

Zusammenfassung. Die Verfasser untersuchten an Hunden die durch enteroceptive Reflexe ausgelösten Blutdruckänderungen. Die Magentraktion, welche unter Chloralosenarkose eine Blutdrucksenkung auslöste, verursachte im Falle der Neuroleptanalgesie II einen Blutdruckanstieg. Die wahrscheinlichste Ursache dieses Unterschiedes ist die Änderung des funktionellen Zustandes der die Spinalreflexbogen beeinflussenden Kortikalgebiete.

Anlaß zu diesen Untersuchungen gaben frühere Beobachtungen [1, 2], die zeigten, daß bei den mit dem Verfahren II der Neuroleptanalgesie anaesthetisierten Patienten die Zahl hypotensiver Zustände — Blutdrucksenkung um mehr als 30% — geringer ist (6,4%) als bei auf andere Weise Narkotisierten (9,6%). Dieser Unterschied war nur bei Bauchhöhlenoperationen bemerkbar [2]. Man kann deshalb annehmen, daß die Blutdrucksenkung durch intraabdominelle chirurgische Manipulation verursacht und der Erfolg der körpereigenen Gegenregulation von der Art der Anaesthesie abhängig ist. Zur Klärung dieser Frage führten wir eine Serie von Tierversuchen durch.

Methode

An 40 Bastardhunden beiderlei Geschlechts wurde in Allgemeinnarkose ein 4 cm breiter Gazestreifen durch die kleine Magenkurvatur gezogen, dessen Ende mittels Rollenübertragung mit 200—500 g belastet wurde. Die Dauer der einmaligen Zugbelastung betrug 20—30 sec. Der Blutdruck wurde blutig über die Arteria femoralis mittels Quecksilber- oder Elektromanometer gemessen und mit dem Kymographen bzw. Polyrecorder registriert.

Ergebnisse

Bei den mit 0,1 g/kg Chloralose narkotisierten Hunden löst der Zug am Magen eine durchschnittlich 21%ige Blutdrucksenkung aus (Abb. 1), welche 10 bis 30 sec lang dauert, der Zugkraft in gewissem Maße proportional ist und sich bei Serienbelastungen wiederholt [4—6]. Dasselbe konnten wir beobachten, wenn wir den Hunden neben Narkosemitteln die Mittel der Neuroleptanalgesie II — Dehydrobenzperidol®, Fentanyl® oder Thalamonal® — einzeln verabreichten. Diese Erscheinung wurde durch die Lähmung der sympathischen α- und β-Rezeptoren nicht beeinflußt.

Wenn wir aber die Hunde mit dem Verfahren der Neuroleptanalgesie II, unter Verwendung von 0,08 mg/kg KG Fentanyl und 2,0 mg/kg KG Dehydrobenzperidol narkotisierten, wurde mit der oben beschriebenen Methode ein *Blutdruckanstieg* ausgelöst (Abb. 2).

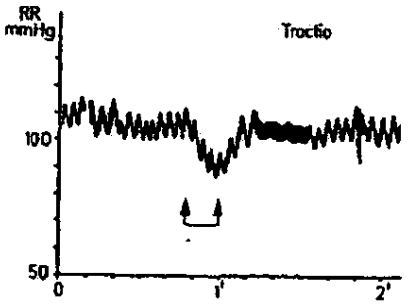

Abb. 1. Zug am Magen löst bei den mit Chloralose narkotisierten Hunden eine Blutdrucksenkung aus

Mitteldruck/mm Hg	98,75	Nach Chloralose	78,25
n	10		10
s_x	2,17		0,79
s		4,53	
t		10,107	
p		$< 0{,}001$	

Besprechung

Zur Erklärung dieser neuen Beobachtung seien hier Untersuchungen von Cyon [8] aus den Jahren 1871—1873 angeführt, der bei peripherer Nervenreizung mit Chloralose narkotisierter Hunde eine Blutdrucksenkung beobachtete. Dasselbe war der Fall, wenn er nach suprabulbärer Decerebration ohne Narkose einen somatischen Reiz auslöste. Wenn er aber bei den mit

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EXHIBIT 51

CLINICAL EXPERIENCES WITH THE NEW CURARE ANTIDOTE GALANTHAMINE (NIVALIN)

E. A. Stojanov

Introduction

In recent times a new curare antidote was added; namely, galanthamine. It is a natural fact that innovations are accepted with enthusiasm by some people and skeptically by others. The skeptics, after getting acquainted with the advantages of innovations, often become enthusiasts. Such is the case with galanthamine. It gained gradually an enviable place in the arsenal of anesthesiologic drugs and is applied today by quite a large number of anesthesiologists in their routine practice.

Galanthaminum hydrobromicum (Nivalin) is an alkaloid of the phenanthridine group containing tertiary nitrogen in its molecule. It was isolated (5) from the blossoms, leaves, and stems of snowdrops (*Galanthus mailis varietas gracilis* and *var. maximus*) growing abundantly in Bulgaria. Galanthamine, from a pharmacological point of view, is an anticholinesterase agent. It potentiates sharply the action of the endogenous acetylcholine which accumulates in the endings of the somatic motor nerves. It does not affect the heart muscle, excluding big concentrations of 10^{-3}, in which case an inotrop action, resembling that of neostigmine, occurs. Galanthamine has also a central stim-

ulating action besides its peripheral anticholinesterase action upon the neuromuscular synapses of the skeletal musculature. It increases the bioelectrical activity of the spinal cord. Its toxicity is low; compared with that of neostigmine it is about 20 times lower.

Mashkowski (3) and Paskov (5) discovered that the properties of galanthamine are similar to those of neostigmine which makes it a reliable antidote of the nondepolarizing muscle relaxants. Consequently, it was introduced in clinical practice. Thorough studies concerning the decurarization possibilities of galanthamine on a large scale (2, 4, 7, 8, 10) followed the first decurarization experiments of Deredjan and Krusteva carried out with several patients (1959). In the course of more than five years galanthamine has become the object of wide clinical testing in the anesthesiology section of the Chair of Surgical Diseases with Urology of the High Medical Institute in Sofia. A number of studies were carried out together with Dr. Mitev from the Chair of Obstetrics and Gynecology of the same Institute and Dr. Jadarni, head of the anesthesiology section of the hospital in Karlovi-Vari, Czechoslovakia, to whom we express our gratitude.

Clinical Studies

The clinical studies comprise 2853 patients; operations being performed are listed in Table I. All age groups from 3 to 99 years are included in Table I and distribution between the sexes was about equal.

The main problem of study was the creation of an exact technique of decurarization, determining the quantitative correlation between the muscle relaxants and galanthamine, the possibilities for coordinating galanthamine with the different anesthetic drugs being examined.

Since Paskov and his collaborators carried out thorough experimental studies of the qualities of galanthamine, our aim was to follow these qualities in the general routine work of the anesthesiologist. The patients were not selected. The object of study was decurarization after administration of nondepolariz-

ing muscle relaxants: d-tubocurarine, gallamine (Flaxedilibe myolan-Spofa) and diallyl-nortoxiferin (Alloferin). The effect of galanthamine in the second phase of action of decamethonium (Procuran-Spofa) was examined in single cases. Ether, halothane (Fluothane), methoxyflurane (Penthrane), cyclopropane, steroid anesthetics (Viadril G), and nitrous oxide were used as anesthetic agents. The preparation of the patients was according to the usual method. In most cases premedication consisted of pethidine (Lydol), promethazine (Prothezin, Phenergan) and Atropinum sulfuricum in corresponding doses.

The technique of decurarization as applied by us lately is very simple. It only differs depending upon whether or not decurarization with galanthamine will include patients in apnea or patients with hypoventilation due to residual curare effect of the used muscle relaxant.

TABLE I. Galanthamine study. Type of operation and number of patients

Thoracic	425
Abdominal	963
Urologic	365
Otorhinolaryngologic	40
Gynecologic	445
Obstetric	130
Traumatologic	280
Orthopedic and others	200
Total	2853

DECURARIZATION OF PATIENTS IN APNEA

The initial dose of galanthamine injected intravenously is between 10 and 15 mg. Nivalin. In most cases we dilute 3 ampules of 5 mg. each with 5–10 ml. physiological saline or 5 percent glucose and inject it slowly within 30–40 seconds through the rubber tube of the infusion drop-system. If breathing is not reestablished sufficiently within 4–5 minutes, a new dose of 10–15 mg. Nivalin is injected. The pulse rate and arterial blood pressure are controlled during this period, spiro-

graphic recording is made, and the quantity of expired air is measured with a volumeter. These two doses are usually enough for reestablishing efficient spontaneous respiration.

DECURARIZATION OF PATIENTS WITH HYPOVENTILATION

Decurarization with Nivalin is carried out according to the action of the muscle relaxants; 10 mg. of the diluted drug is enough in most cases, but if we assess that the curare block is not adequately eliminated, a dose of 10 mg. Nivalin may follow.

We may talk to the patient 3–5 minutes after decurarization. He obeys our orders and gives answers when asked.

Effect of galanthamine decurarization on blood circulation. No sharp fluctuations in frequency of pulse rate were observed. There were no changes in the pulse rate in 60 percent of decurarized patients. A decrease of 5–15 beats per minute occurred in some patients and in very few cases the pulse rate was accelerated. An intravenous injection with atropine was applied only in two patients on account of a slow pulse rate up to 56 beats per minute due to inconsidered sinus bradycardia.

In most of our patients no changes in the arterial blood pressure were observed, as is also the case with the pulse rate. In a few cases a reduction of 10–15 mm. was found which was restored to normal levels in a short period of time.

The cardiac rhythm in all of the patients remained constant. Many of our patients had serious heart conditions. Therefore, from the very beginning of our tests we studied the effect of galanthamine in 40 patients with hypoxia of the myocardium and myocardial lesions. The cardiac activity and its rhythm remained unchanged on decurarization with Nivalin.

The secretion of saliva, sweat, and bronchial secretions were carefully followed after decurarization with galanthamine. Salivation increased in most of the patients only when the quantity of administered Nivalin exceeded 30–40 mg. Injection of 0.5 mg. atropine was necessary in four patients to depress the increased salivation. Bronchial secretions did not increase.

Perspiration with moistening of the skin was observed only occasionally.

Atropine is not compulsory before decurarization with Nivalin due to its slight muscarinic effect. No atropine was given to most of our patients. The exceptions were patients with sinus bradycardia before operations or patients with a pulse rate under 64 per minute prior to decurarization. Prophylactic atropinization with 0.5 or 0.25 mg. of atropine was only used to avoid the increase of salivation when injection of more than 30–40 mg. galanthamine was necessary. According to Bergmann, atropine may cause postoperative intestinal pareses. Therefore, galanthamine has considerable advantages compared with other decurarizing drugs.

The spirographic method was applied to 80 patients in order to study more precisely the features of galanthamine decurarization. It was established that Nivalin may reverse the action of nondepolarizing muscle relaxants even in apneic patients when it is injected at the end of operation and narcosis. The first spontaneous respiratory movements appear after an interval of one minute up to one minute and 40 seconds from its application. This time depends upon the kind of muscle relaxant and anesthetics applied. Following d-tubocurarine the first breath is taken after one minute and 20 seconds on the average. The antidote power of Nivalin against Flaxedil is greater. The reversal effect is accomplished within 40 seconds to one minute. If Nivalin is given after diallylnortoxiferine, breathing reappears within 1½ to 2 minutes. The maximal action in relation to breathing appears after 3–5 minutes. Respiration gradually intensifies after the first breathing movements and becomes rhythmical. Tachypnea may occur in some cases when respiration accelerates up to 48 per minute. It normalizes gradually in a short period of 2–3 minutes.

Galanthamine leads to a quick reversal of muscle relaxation in all patients with residual effect of the relaxants when breathing is still inadequate. Respiration is intensified within less than a minute, the maximal effect appearing between one and two minutes after the injection of galanthamine.

We tried to establish spirographically the quantitative correlations between the nondepolarizing relaxants and galanthamine. We found that 20–30 mg. Nivalin are necessary for eliminating the developed action of 10 mg. d-tubocurarine. Concerning gallamine this correlation is 1:2; i.e., 20 mg. gallamine

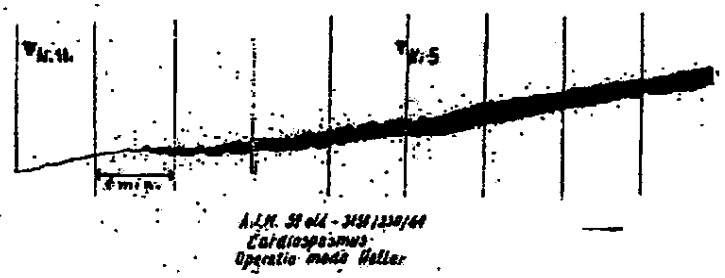

FIGURE 1. Decurarization of patient in apnea. Spirographic tracing. At Ni 10, 10 mg.; at Ni 5, 5 mg. galanthamine were administered i.v.

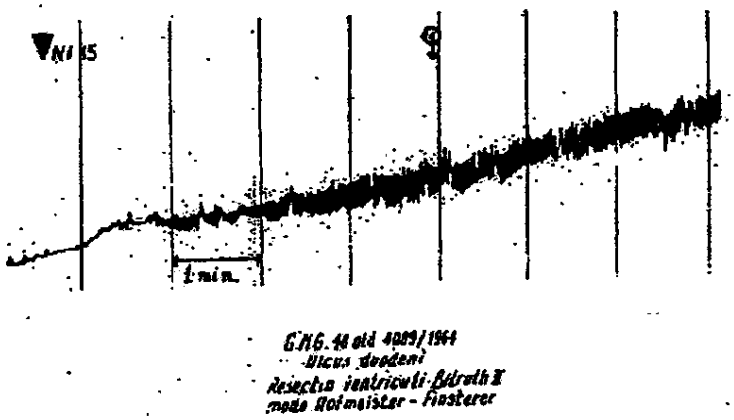

FIGURE 2. Decurarization of patient with residual effect of relaxant. Spirographic tracing. At Ni 15, 15 mg. galanthamine were administered i.v.

may be antagonized up to 5 minutes with 10 mg. Nivalin. Naturally the breakdown of the relaxant in the organism is not considered here since only several minutes elapse from the moment of applying the relaxant up to decurarization. This correlation, however, is different in the actual clinical practice.

The reverse study—injecting the muscle relaxant after Nivalin—shows a complete loss of the relaxing activity of d-tubocurarine and gallamine.

The degree of decurarization with Nivalin and neostigmine was comparatively studied in two groups of 50 patients. The relative standard of the terms of study was ensured by an approximately equal degree of respiratory depression before injecting one of these drugs. It was established that the decurarization effect of 1 mg. neostigmine corresponds to the action of 15–20 mg. Nivalin. Full progress of the depolarization effect of Nivalin advances more slowly compared with neostigmine. Here we consider only patients whose respiration was already partially restored. In apneic patients decurarization with neostigmine was far slower and less efficient than with Nivalin. Considering the duration of action of different doses of Nivalin, we found that it was much longer lasting than neostigmine, i.e., between two and three hours. Not a single case of recurarization was observed in our large series of patients. No patients resistant to Nivalin were encountered. The largest quantity of Nivalin given to one single patient was 60 mg. Rusev (6) reports decurarization with 80 mg. Nivalin administered to a patient after an operation of fossa crani posterior. With neostigmine we are not sure whether we have not encountered patients who were resistant to it. Twice during the period when galanthamine was not available and decurarization was accomplished with neostigmine (8) we could not achieve full decurarization. One of these patients died and the normal breathing of the second was restored only after 11 hours, in spite of the obstinate efforts to accomplish decurarization with neostigmine.

Volumetric studies were carried out in 320 patients which confirmed the data of spirographic recordings. The first respiratory movements appeared after 1 minute and 30 seconds. The respiratory volume, quantity of exhaled air, was between 50 and 100 ml. at the beginning but not seldom patients returned from apnea with their first inhalation to a respiratory volume of 200–250 ml. The respiratory volume increased by 50 to 75 ml. on

the average in every subsequent minute. The maximal effect of Nivalin leading to normalization of the minute respiratory volume, the tidal volume, and respiratory rate preceding narcosis was encountered between three and six minutes after administration of galanthamine (Figure 3). This was achieved even

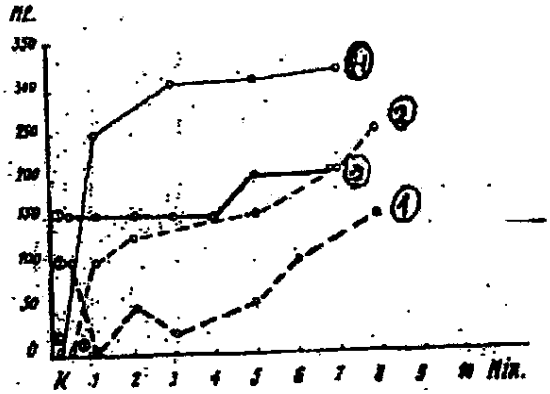

FIGURE 3. Measurements of tidal volume (expiratory) in milliliters following i.v. injection of an antidote at K. All patients were of the same age, anesthetized with ether, and relaxed with equal doses of *d*-tubocurarine. Patients 1 and 3 received neostigmine; patients 2 and 4 received equivalent doses of galanthamine.

faster in patients with partially restored respiration. No abbreviations of the results attained at the beginning were encountered in the control studies of tidal and minute respiratory volume made with some of the patients 30 minutes, one hour, and one hour and 30 minutes after decurarization. The results attained during the first minutes in the control group of patients decurarized with neostigmine were not constant. They decreased after 8–10 minutes.

In galanthamine decurarization we observed a characteristic feature lacking all other curare antidotes; namely, considerable vigor and quick reestablishment of respiratory reflexes (Figure 4). Patients could talk after decurarization and intensified their respiration upon command. Coughing reflexes were fully reestablished very quickly.

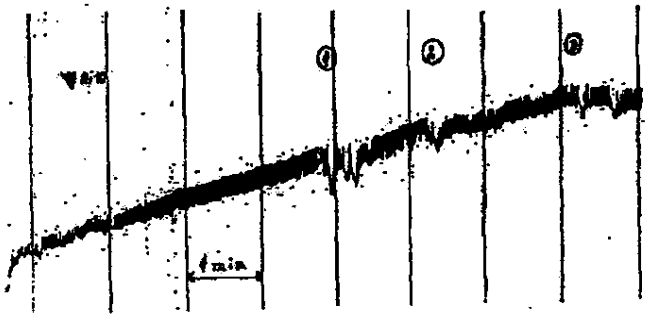

FIGURE 4. Spirographic tracing of patient who had received 10 mg. of galanthamine at Ni 10. At 1, 2, and 3, he was asked to take deep breaths.

As we know, the action of muscle relaxants is influenced by anesthetic agents. Most anesthetics prolong the action of curare preparations. Vigilance is recommended in decurarization with a number of anesthetics due to possible danger of complications in relation to blood circulation and respiration. For this reason, we traced the influence of galanthamine decurarization in patients anesthetized with various agents (Figure 5): ether, methoxyflurane, halothane, hydroxydion, cyclopropane, and nitrous oxide. Nivalin may be used with all these agents. We established, in collaboration with Dobrev and

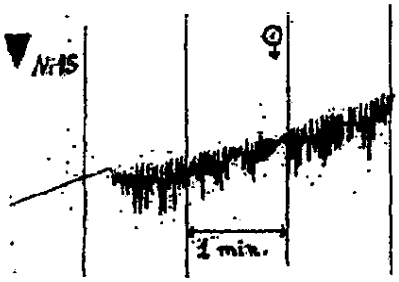

FIGURE 5. Spirographic tracing of 64-year-old patient following curarization under ether anesthesia. At Ni 15, 15 mg. galanthamine were given i.v. followed by prompt return of spontaneous respiration. At 1, patient was asked to breathe deeply.

Paskov (9) that galanthamine is a good antidote for hydroxydion and its respiratory depressing effect. Decurarization with Nivalin proceeds well and without complications concerning all anesthetic agents studied by us.

Most of our patients were older than 60 years. Old people endure well the decurarization with galanthamine. They awoke at stated intervals and their muscular tonus was restored. Galanthamine decurarization is also well tolerated by children.

Apart from patients with heart conditions, decurarization with Nivalin was applied also to patients with diabetes and kidney diseases. We stimulated respiration with Nivalin after extirpation of thymus tumors and hyperplastic thymus glands. No damage to the liver, water-mineral balance, and uropoiesis, or hemopoiesis were established due to the application of galanthamine. We used Nivalin for stimulation of intestinal peristalsis by intramuscular injection of 5 mg. twice a day.

Discussion and Summary

Decurarization is one of the problems of contemporary anesthesia which is to be thoroughly studied in the future. One of its main weak points is that the agents used as antidotes of the muscle relaxants do not solve the problems posed to them. It is very important in our routine practice to restore normal respiration in the shortest possible time following narcosis. The most crucial moment is when complete reversal of relaxation is imposed. Then in fact the organism must again restore its normal physiologic status from an unnatural state—an action connected with many dangers. Concerning the requirements of an antidote to muscle relaxants and on the basis of our clinical studies, we may state that galanthamine has the following principal advantages:

1. It abolishes the relaxant action of the nondepolarizing muscle relaxants. Decurarization proceeds calmly with no vigorous changes in blood circulation. It may be applied in patients with heart conditions and other accompanying diseases.

2. Its muscarinic effect is slight. No obligatory preliminary atropinization of patients is needed.
3. The anticurare action of galanthamine lasts between two and three hours. It may be applied in the early postanesthetic and postoperative period; in which case, besides its anticurare action, it also stimulates intestinal peristalsis.
4. Of special value is its action on the central nervous system and the quick recovery of reflexes of the upper respiratory tract. As far as opiates and steroid anesthetics are concerned, the antidote action of galanthamine also eliminates their residual depressive effect on respiration.

Galanthamine leads the way for a new type of drug in anesthesiology. In the near future, no doubt, it will be the initial product of other curare antidotes, opiates, and some anesthetics. They will help us to decrease and avoid still existing and frequently displayed dangers.

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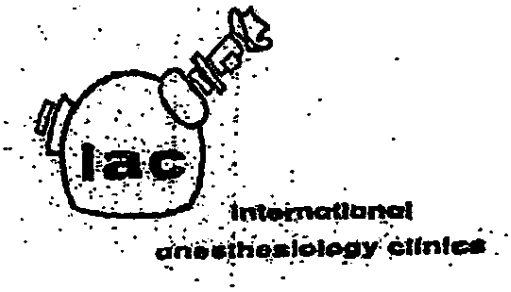

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EXHIBIT 52

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EXHIBIT 53

REDACTED

EXHIBIT 54

CURRICULUM VITAE

Name: Joseph T. Coyle, M.D.

Address: 115 Mill Street, Belmont, MA 02478

Place of Birth: Chicago, Illinois

Marital Status: Married, 1968; Genevieve Sansoucy Coyle
Children: Peter Joseph, Andrew Jerome and David Sansoucy

Education:

1965 A.B.	*in cursu honoris cum laude*; College of the Holy Cross
1969 M.D.	The Johns Hopkins University School of Medicine

Postdoctoral Training:

Internship and Residencies:

1969-1970	Intern in Pediatrics, The Johns Hopkins Hospital
1973-1976	Resident in Psychiatry, The Johns Hopkins Hospital

Research Fellowships:

1970-1973	Research Associate, Laboratory of Clinical Science, National Institute of Mental Health (Dr. Julius Axelrod), Bethesda, Maryland
2001	Neurobiology, Marine Biological Laboratory, Woods Hole, MA

Licensure and Certification:

1970	Maryland License Registration D15842 (inactive)
1970	Diplomate - National Board of Medical Examiners
1980	Board Certified in Psychiatry by the American Board of Psychiatry and Neurology
1991	Massachusetts License Registration 75163

Academic Appointments:

1974-1976	Assistant Professor of Pharmacology, The Johns Hopkins University School of Medicine
1976-1978	Assistant Professor of Pharmacology and Psychiatry, The Johns Hopkins University School of Medicine
1978-1980	Associate Professor of Pharmacology and Psychiatry, The Johns Hopkins

	University School of Medicine
1980-1991	Professor of Neuroscience, Psychiatry and Pharmacology, The Johns Hopkins University School of Medicine
1982-1991	Director: Division of Child Psychiatry, Professor of Psychiatry, Neuroscience, Pharmacology and Pediatrics, The Johns Hopkins University School of Medicine

Academic Appointments continued:

1985-1991	Distinguished Service Professor of Child Psychiatry, The Johns Hopkins University School of Medicine
1991-2001	Chair of the Consolidated Department of Psychiatry, Harvard Medical School
1991-	Eben S. Draper Professor of Psychiatry and of Neuroscience, Harvard Medical School

Endowed Lectureships and Major Visiting Appointments:

1981	Smith, Kline and French Visiting Professor, Flinders University and University of New South Wales, Australia
1983	Harold C. Voris Memorial Lecturer in Neuroscience, Mercy Hospital, University of Illinois School of Medicine, Chicago
1983	Grass Lecturer, University of Missouri School of Medicine
1986	Dean's Lecture, The Johns Hopkins School of Medicine
1987	Sterling Drug Visiting Professor, Department of Pharmacology, Medical College of Virginia
1987	Pfizer Visiting Professor of Psychiatry, Columbia University College of Physicians and Surgeons
1987	Tarbox Distinguished Neuroscientist Lecturer, Texas Tech University
1987	Grass Lecturer, University of South Carolina School of Medicine
1988	Nielson Lecture, University of Utah School of Medicine
1989	Centennial Visiting Professor, Celebration of the Sciences Lecturer, Washington College
1989	Dean's Distinguished Lecture, University of Colorado School of Medicine
1989	Halbert Robinson Distinguished Lecturer, University of North Carolina School of Medicine
1991	Axelrod Lecturer, City College of New York
1991	Gerard Symposium, University of Michigan
1992	Meyerowitz Lecturer, University of Rochester
1993	Andrew Woods Visiting Professorship and Lecture, University of Iowa
1993	Harvey Shein Lecture, American Association Psychiatric Residency Training Directors
1993	John E. Whitmore Lecture, Baylor College of Medicine
1993	The Wesco Lecture, University of Kansas School of Medicine
1993	Thomas Salmon Lecture, New York Academy of Medicine

1993	Jonathan Swift Psychiatric Lecture, St. Patrick's Hospital, Dublin, Ireland
1993	The Taylor Lecture in Neurology and Psychiatry, University of Maryland School of Medicine
1993	Pfizer Visiting Professor of Psychiatry, Allegheny General Hospital
1994	Adolf Meyer Lecture, American Psychiatric Association
1995	Guildea Lecture, Washington University School of Medicine
1995	Harold E. Cooper Lectureship, University of Texas Medical School
1995	Jonathan Cole Lectureship, St. Elizabeth's Hospital and Tufts University School of Medicine
1996	Ribicoff Lecture, Yale University School of Medicine
1996	Thomas L. O'Donohue Memorial Lecture in Neuropharmacology, Howard University College of Medicine
1997	Pfizer Visiting Professor of Psychiatry, University of North Carolina School of Medicine

Endowed Lectureships and Major Visiting Appointments continued:

1998	Seventy-Seventh Annual Beaumont Lecture, Wayne County Medical Society
1999	Stephen R. Max Memorial Lecture, University of Maryland School of Medicine
1999	Deane Lecture, Wellesley College
1999	Leo Kanner Lecture in Child and Adolescent Psychiatry, Johns Hopkins University School of Medicine
1999	Pfizer Visiting Professor of Psychiatry, Jefferson Medical College of Thomas Jefferson University
1999	Margaret Roche Donlon Bidwell Memorial Lecture, Massachusetts Institute of Technology
2000	The Distinguished Lecture in Neuroscience, University of Texas Medical School at Houston
2001	Kwin Finnegan Memorial Lectureship, University of Utah School of Medicine
2002	Lucile Packard Distinguished Lecture, Stanford University Medical Center
2002	The Twenty-Third Annual Alberto DiMascio Memorial Lecture, Tufts University School of Medicine
2005	Janssen Visiting Professor of Psychiatry, University of Washington School of Medicine
2005	Frederick G. Corneel Memorial Lecture, McLean Hospital
2006	Morris H. Aprison Lecture, Indiana University School of Medicine

Awards and Honors:

1968-1969	Henry Strong Denison Research Scholarship

1969	Alpha Omega Alpha Student Research Award
1972	Fellowship for Third Study Program for Neurosciences Research Program, Boulder, Colorado
1977	Basil O'Connor Award from the March of Dimes
1977-1987	National Institute of Mental Health Research Career Development Award, Type II
1978	A.E.Bennett Award in Basic Science from the Society of Biological Psychiatry
1979	John Jacob Abel Award from the American Society of Pharmacology and Experimental Therapeutics
1979	Sato International Memorial Award from the Japanese Pharmaceutical Society
1982	Daniel Efron Award from the American College of Neuropsychopharmacology
1985	Foundations' Fund Prize for Research in Psychiatry, American Psychiatric Association
1985-1991	Javits Neuroscience Investigator Award, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health
1986	Alpha Omega Alpha Honor Society
1990	Nancy and Daniel Weisman Award for Research on Mental Retardation
1990	Institute of Medicine of the National Academy of Sciences
1991	Edward A. Strecker Award from the Institute of Pennsylvania Hospital
1991	The Gold Medal Award from the Society of Biological Psychiatry

Awards and Honors continued :

1991-92	President, Society of Neuroscience
1992	William R. McAlpin, Jr. Research Achievement Award from the National Mental Health Association
1994	Fellow, American Academy of Arts and Sciences
1995	Hilton Investigator Award from the National Alliance for Research on Schizophrenia and Depression
1996	Kempf Fund Award for Research Development in Psychobiological Psychiatry, American Psychiatric Association
1997	Robert J. and Claire Pasarow Foundation Award for Neuropsychiatric Research
1997	Exemplary Psychiatrist Award, National Alliance for the Mentally Ill
2001-02	President, American College of Neuropsychopharmacology
2001-04	Council, American Association for the Advancement of Science
2001	Highly Cited Researchers Award, ISI Thomson Scientific
2001	Society for Neuroscience, Special Achievement Award
2003	Organizer and Chairman (with C. Robert Cloninger) "Integrating Programs with Genetics and Neuropharmacology of Schizophrenia," Cold Spring Harbor Laboratory

2004	Lieber Prize, National Alliance for Research on Schizophrenia and Depression
2005	Society for Neuroscience, Award for "Lifelong Dedication to Excellence and Diversity in Neuroscience"
2005	Elected Fellow, American Association for the Advancement of Science
2006	Sanctae Crucis Award, College of the Holy Cross

Major Committee Assignments:

National Institute of Mental Health:

1977-1981	Preclinical Psychopharmacology Research Review Committee Member
1982	Scientific Councillor for the Intramural Program, ad hoc
1985-1989	Cellular Neurobiology and Psychopharmacology Research Review Committee, Chairman
1985-1988	Extra-mural Scientific Advisory Board
1990-1994	National Advisory Mental Health Council
1995	Search Committee for NIMH Director

Institute of Medicine:

1988	Committee on Research on Children and Adolescents with Mental, Behavioral and Developmental Disorders
1989	Committee on a National Neural Circuitry Database
1992	Committee on Prevention of Mental Disorders
1993-1995	Membership Committee
1991-2000	Board of Biobehavioral Sciences and Mental Disorders
1994-2000	Chair, Board of Biobehavioral Sciences and Mental Disorders

The Johns Hopkins University School of Medicine:

1979-1991	Graduate Education Steering Committee, Department of Pharmacology
1979-1982	Member: Medical School Council
1981-1982	Vice Chairperson: Medical School Council
1981-1982	Member: Advisory Board to the School of Medicine
1990-1991	Pharmacy and Therapeutics Committee
1981-1991	Medical Scientist Training Program Steering Committee

Major Committee Assignments continued:

1978-1982	Director: Interdisciplinary Postdoctoral Training Program in Neurosciences, MH-15330
1983-1986	Johns Hopkins University Press, Editorial Board
2002-2005	Working Group on Interspecific Chimeric Primate Brains

Harvard Medical School:

1991-1994	Subcommittee of Professors
1991-1993	Committee of Professors
1991-1996	Harvard Medical Center, Board of Trustees

1992-1996 Graduate Medical Education Committee
1994-1995 HMS Research Council
1994 LCME Self Study Committee on Objectives, Chairman
1996 Search Committee for Dean Harvard Medical School

Memberships, Offices and Committee Assignments in Professional Societies:

Society for Neuroscience (Member 1975):
1980-1983 Program Committee
1986 Chairman of Program Committee, Washington, D.C. Meeting
1986-1988 Council
1988-1989 Treasurer
1989-1990 Council
1990-1991 President Elect
1991-1992 President
1995-2003 Chairman, Governmental and Public Affairs Committee
1995-2005 Deputy Director, Minority Neuroscience Fellowship Program

1975- American Society for Neurochemistry

American Association for the Advancement of Science
2001-2004 Neuroscience Section: Council Delegate
2001-2004 Council Affairs Committee

American Psychiatric Association (Member 1976):
1990-2003 Fellow
2003-2006 Distinguished Fellow
1986-1990 Scientific Advisory Panel
2000- Institute for Research and Education Scientific Advisory Panel
1976 Sigma Xi
2006- Distinguished Life Fellow

1978- American Society for Pharmacology and Experimental Therapeutics

Collegium Internationale Neuropsychopharmacologicum (Member 1978):
1997-1998 Nominating Committee
2001-2004 Program Committee

American College of Neuropsychopharmacology (Member 1979, Fellow 1997)
1989-1991 Finance Committee
1991 Awards Committee

Memberships, Offices and Committee Assignments in Professional Societies continued:

1991- Journal Editorial Board- Neuropsychopharmacology

1996-1998 Committee on Problems of Public Concern
1997-1999 Program Committee (Co-Chair)
1998-2001 Council
2001-2002 President
2002-2004 Council
2004- Publication Committee (Co-Chair)

1980 National Foundation March of Dimes, Scientific Advisory Board, ad hoc
1981-1985 National Huntington's Disease Association National Medical and Scientific Advisory Council
1982-1986 Committee to Combat Huntington's Disease, Scientific Advisory Board
1982- International Society for Developmental Psychobiology
1982-1990 Hereditary Disease Foundation, Scientific Advisory Board

Alzheimer's Disease and Related Disorders Association
1982-1985 Scientific Advisory Board
1990-1992 Scientific Advisory Board

American Academy of Child and Adolescent Psychiatry (Member 1982):
1989-1992 Work Group on Research

1985-1988 International Rett's Syndrome Association, Professional Advisory Board
1988-1993 Pfizer Scholars Award, Advisory Board
1990-2003 John F. Merck Foundation, Scientific Advisory Board
1991- Massachusetts Hospital Association
1991- Massachusetts Psychiatric Society
1993-2001 Board of Trustees, McLean Hospital
1994- National Alliance for Autism Research Scientific Advisory Board
1995- Health Emotions Research Institute Scientific Advisory Board
1995- American College of Psychiatrists (Fellow)
1995- Dana Alliance for Brain Inititatives, Scientific Advisory Board
1996-1998 Hitchings-Elion Fellowships/Wellcome Research Travel Grant Advisory Committee
1996- International Academy for Biomedical and Drug Research
1996- Board of Trustees, Judge Baker Children's Center
2001- Marine Biological Laboratory Alumni Relations Advisory Board
2002-2004 Research Advisory Committee on Gulf War Veterans' Illnesses

Major Research Interest:

Signal Transduction in the Nervous System

Editorial Responsibilities:

1993-2003	Harvard Review of Psychiatry: Editor-in-Chief
2002-	Archives of General Psychiatry: Editor-in-Chief

Editorial Responsibilities Continued:

Editorial Boards:

1984-	Journal of Developmental and Behavioral Pediatrics
1984-	Developmental Brain Research
1984-1992	Neuropharmacology
1986-1992	European Journal of Pharmacology
1988-1993	The Journal of Neuroscience
1988-	Molecular Psychiatry
1988-	Molecular and Chemical Neuropathology
1988-	Synapse
1989-	Archives of General Psychiatry
1989-1999	Metabolic Brain Disease
1990-	Journal of Neuroscience Research
2000-	Acta Paedopsychiatrica
1991-2000	Journal of Child and Adolescent Psychopharmacology
1991-	Neuropsychopharmacology
1992-1998	Advances in Pharmacology
1992-1998	Oxford University Press Psychiatry Series
1992-	Neurobiology of Disease
1993-	Cerebral Cortex
1993-2000	Current Opinion in Psychiatry
1994-	Neuroscience
1994-	Journal of Psychiatric Research
1995-2001	American Psychiatric Press, Inc.
1996-	Acta Paedopsychiatrica, International Journal of Child and Adolescent Psychiatry
1996-2002	Journal Watch for Psychiatry
1997-2001	American Journal of Psychiatry
1999-	Journal of Molecular Neuroscience
2002-	Journal of the American Medical Association

Advisory Boards:

1978-1981	Developmental Neurosciences
1979-	Life Sciences
1983-	Neurobehavioral Toxicology
1984-1993	Neurobiology of Aging
1991-	CRC Critical Reviews in Neurobiology

1992-1996	Cambridge Series in Psychopharmacology
1995-	Current Protocols in Pharmacology
1999-	The Autism Brain Library Trust

Doctoral Students and Titles of Theses:

Kathleen Bizière, M.D., Ph.D. (1978) "Etude d'un modèle animal de la Chorée de Huntington"

Michael McKinney, Ph.D. (1982) "Cholinergic Innervation of the Mammalian Cerebral Cortex and Hippocampus by the Basal Forebrain: Implications for Senile Dementia of the Alzheimer Type"

Alfred Malouf, Ph.D. (1983) "The Regulation of [3H]Glutamic Acid Binding Sites on N18-RE- 105 Neuroblastoma Hybrid Cells in Culture"

Kerry Koller, Ph.D. (1984) "The Purification and Pharmacologic Characterization of N-Acetyl-Aspartyl-Glutamate, a Possible Excitatory Neurotransmitter"

Robert Zaczek, Ph.D. (1986) "Characterization of a Novel Brain Specific Chloride Dependent Glutamic Acid Transport"

Randy D. Blakely, Ph.D. (1987) "N-Acetyl-Aspartyl-Glutamate: The Elucidation of Specific Catabolic and Anatomic Pathways in the Rat CNS"

Timothy H. Murphy, Ph.D. (1989) "Glutamate Toxicity in a Neuronal Cell Line Involves Inhibition of Cystine Uptake Leading to Oxidative Stress"

Mario D. Saltarelli, M.D., Ph.D. (1989) "Studies on the Regulation of High-Affinity Choline Uptake"

Joanne E. Sweeney, Ph.D. (1989) "Developmental, Neurochemical and Functional Properties of the Cholinergic Basal Forebrain Complex in Mice"

Barbara Stauch Slusher, Ph.D. (1991) "Purification, Antibody Production, and Immunocytochemical Localization of Rat Brain N-Acetylated α-Linked Acidic Dipeptidase (NAALADase)"

Guochuan Tsai, M.D., Ph.D. (1991) "Anatomy, Physiology and Pathophysiology of N-Acetylaspartylglutamate"

Carter, Ruth, Ph.D. (1997) "Cloning and Expression of the Naladase Neuropeptidase"

Passani, Lucius, Ph.D. (1997) "Distribution of N-acetylaspartylglutamate and N-acetylated Alpha Linked Acidic Dipeptidase in Human Brain and the Effects of Disease Related and Induced Neuronal Degeneration"

Schwartz, Paul J., Ph.D. (1998) "Effect of Altered Expression of the Cytoplasmic Copper-Zinc Superoxide Dismutase on Oxidative Stress Mediated Phenomena: Implications for Down's Syndrome and Glutamate Neurotoxicity"

Post-doctoral Fellows and Current Positions:

Reinhard Grzanna, Ph.D. 1975-78, RMG Biosciences, Baltimore

Robert Schwarcz, Ph.D., 1976-78, Professor of Psychiatry and Pharmacology, University of Maryland School of Medicine

Edythe London, Ph.D., 1978-80, Professor of Psychiatry, UCLA

John Slevin, M.D., 1979-81, Professor of Neurology, University of Kentucky

Michele Beaulieu, Ph.D., 1980-82, Hoffman-LaRoche

Michael Johnston, M.D., 1980-82, Professor of Neurology, Johns Hopkins

Larry Tune, M.D., 1980-82, Professor of Psychiatry, Emory University

Peter Campochiaro, M.D., 1982-83, Professor of Opthalmology, Johns Hopkins

Paul Sanberg, Ph.D., 1982-84, Professor of Neurosurgery, University of Florida Medical School
John Lehmann, Ph.D., 1983-85, Associate Professor of Neuroscience, Hahnemann Medical School
Pedro Lowenstein, M.D., Ph.D., 1985-87, Professor of Neuroscience, University of California, Los Angeles
Michael Robinson, Ph.D., 1986-89, Professor of Pediatrics and Pharmacology, University of Pennsylvania School of Medicine
Giancarlo Forloni, Ph.D., 1986-88, Mario Negri Institute, Milan

Post-doctoral Fellows and Current Positions Continued:

Piero Antuono, M.D., 1985-87, Associate Professor of Neurology, Medical College of Wisconsin
Carla Bendotti, Ph.D., 1986-88, Mario Negri Institute, Milan
Christine Hohmann, Ph.D., 1987-90, Professor of Biology, Morgan State University
George Capone, M.D., 1988-90, Associate Professor of Pediatrics, Johns Hopkins
Marilyn Saunders, M.D. 1987-89, Associate Professor of Pediatrics, University of Conneticut School of Medicine
Pamela Puttfarcken, Ph.D., 1989-92, Scientist, Abbott Laboratories
Maria Caserta, M.D., Ph.D., 1989-91, Associate Professor of Psychiatry, Northwestern University School of Medicine
James Vornov, M.D. Ph.D. 1987-91, Guilford Pharmaceuticals
Robert Tasker, M.D., Ph.D., 1989-91, Dept of Pediatrics, University of Cambridge
Urs Berger, Ph.D., 1992-94, Instructor in Psychiatry, Harvard Medical School
Guochuan Tsai, M.D., Ph.D., 1994-95, Associate Professor of Psychiatry, UCLA School of Medicine
Richard Bergeron, M.D., Ph.D., 1995-2000, Assistant Professor of Psychiatry, University of Ottawa School of Medicine
Cecelia Flores, Ph.D., 2000-02, Instructor, Montreal Neurological Institute
Jonathan Pickar, M.D., Ph.D., 2003-06, Instructor in Medicine, Harvard Medical School
Alo Basu, Ph.D., 2005-, Research Fellow, Harvard Medical School
Amy Lawson-Yuen, M.D., Ph.D., 2005-, Reseaarch Fellow, Harvard Medical School
Micheal Benneyworth, Ph.D. 2007-, Post-doctoral Fellow

Employment Resumé:

Dr. Coyle was appointed an Assistant Professor of Pharmacology while a Resident in Psychiatry at Johns Hopkins Medical School in 1974. Four years after completing his Residency in Psychiatry, he was promoted to the rank of Professor of Pharmacology and of Psychiatry in 1980.

In 1981, he was named Professor and Director of the Division of Child and Adolescent Psychiatry at Johns Hopkins. At the time, the Division consisted of four junior faculty members, had no external research support, trained one to two residents per year and provided care in outmoded facilities. During his nine years as Director, the Division developed 26 bed inpatient service for child and adolescent patients, increased the Residency Training Program to five positions per year, expanded to ten faculty members and attracted nearly $2MM per year in grant and contract support. Several faculty then recruited to the Division now hold leadership positions at their institutions including the Directors of the Division of Child and Adolescent Psychiatry at Stanford (A. Reiss, M.D.), at George Washington (P. Joshi, M.D.) and at Jefferson (G. Edelsohn, M.D., M.P.H.) and the Director of the Mental Retardation Research Institute of the University of North Carolina (J. Piven, M.D.).

In 1991, he was recruited to Harvard Medical School to serve as the Chairman of five of its nine affiliated programs in Psychiatry. In three years, the remaining affiliated programs in Psychiatry joined the Consolidated Department of Psychiatry, making it the only academically unified major clinical department at Harvard Medical School. The Department contained over 1500 part-time and full-time faculty and has nearly 200 residents in adult and child psychiatry in training. He reorganized residency training, condensing six competing adult residency training programs into three thematically differentiated programs with a single application form. The six child psychiatry residencies were merged into three with a single core curriculum. The Medical Student curriculum, historically dependent on idiosyncrasies of the nine hospital departments, was reorganized with clear objectives so that all students could be subject to the same tests of their knowledge. He focused on the career development of women, resulting in substantial increases in women's representation at the assistant and associate professor levels and a four-fold increase in women professors. Between 1991 and 2001, the external funding for the components of the Consolidated Department of Psychiatry grew from less than $20 MM to over $65MM. Through outreach efforts with the Department of Mental Health of the State of Massachusetts, the Consolidated Department of Psychiatry received an annual grant of nearly $3MM per year to support residency education in Psychiatry. In addition, the Department received $2.2 MM per year from the State for a 12-bed inpatient unit to carry out clinical research on severe mental illness. He stepped down as Chairman in 2001 after ten years and holds the Eben S. Draper Chair of Psychiatry and Neuroscience.

Dr. Coyle's research in neuroscience has been continuously funded by NIH since 1975, and he currently serves as the Director of a $9 MM NIMH Conte Center on the Neurobiology of Schihzophrenia. He has also played a national leadership role in Neuroscience and Psychiatry. He served as Councilor, Treasurer and ultimately President (1991-92) of the Society for Neuroscience, an international scientific organization with over 30,000 members. He served on an NIMH Initial Review Group (IRG) for eight years, four of which he was the chairman. He also served on the National Advisory Council to NIMH (1990-94). Elected to the Institute of Medicine in 1990, he chaired the Board of Neuroscience and Behavioral Health (1994-2000). He was president of the American College of Neuropsychopharmacology (ACNP), a leading honorific society in Psychiatry, in 2002. He sits on the editorial advisory boards of over twenty journals including *JAMA* and is the editor-in-chief of the *Archives of General Psychiatry*, the most highly cited journal in the field. He was elected a fellow of the American Academy of Arts and Sciences and of the American Association for the

Advancement of Science. His research publications have been cited >27,000 times in the scientific literature. .

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